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EFFECT OF SOIL-MOISTURE CONTENT ON THE RATE OF PHOTOSYNTHESIS AND RESPIRATION IN LADINO CLOVER (TRIFOLIUM REPENS L.) [1,2]

ROBERT P. UPCHURCH,[3] MAURICE L. PETERSON AND ROBERT M. HAGAN

DEPARTMENTS OF AGRONOMY AND IRRIGATION, UNIVERSITY OF CALIFORNIA, DAVIS, CALIFORNIA

Numerous studies have been conducted to evaluate the effect of variations in soil-moisture on plant behavior. The objective of many of these investigations has been to determine the most suitable irrigation practices for obtaining good yields and quality in crop plants. Relatively few investigators have attempted to examine the effects of soil moisture treatments upon particular physiological processes.

The present investigations on ladino clover were undertaken to determine the effects of variations in soil moisture on the rates of photosynthesis and respiration as measured by carbon dioxide exchange. This information should help explain the responses of this forage plant to different irrigation treatments under field and laboratory conditions. The latter studies have been in progress at the California Station since 1948.

The physiological responses of plants to variations in soil-moisture contents have been reviewed by Veihmeyer and Hendrickson (12) and by Richards and Wadleigh (9). The latter reviewers conclude that soil-moisture conditions may have a major effect upon the process of photosynthesis. These reviews also indicate the difficulty in making a simple general statement which describes the physiological responses of plants to variations in soil-moisture content between field capacity and the permanent wilting percentage.

Three publications (1, 6, 10) are of primary importance to the discussion of the present work. Schneider and Childers (10) determined the net CO_2 exchange by attached leaves of small apple trees as the soil in which they were grown was allowed to dry to the wilting percentage. Experiments were conducted both in the field and under controlled environmental conditions. They report that before wilting was evident, there were marked reductions in apparent photosynthesis and transpiration and an increase in respiration. Other work (2, 5) tends to support these conclusions.

Allmendinger et al (1) studied the effect of soil-moisture content on the net CO_2 exchange of apple leaves over a 4-week period. Plants were allowed to extract 20, 40, 60, 80 and 100 % of the available soil moisture and were then watered to field capacity. They write, "Apple trees growing in the greenhouse did not show a reduction in apparent photosynthesis until more than four-fifths of the available soil water had been utilized." Actually the reduction in apparent photosynthesis occurred when about 10 % of the available soil moisture remained. Yet, they reported that the growth of trees as measured by terminal elongation of branches was reduced when the plants were allowed to extract 80 % of the available soil moisture.

Loustalot (6) grew pecan trees in soil containers and measured the CO_2 exchange by parts of certain leaves while the plants were allowed to extract the soil moisture from field capacity to the permanent wilting percentage. No environmental controls were used in this investigation. He found that a more complete exhaustion of soil moisture was required to bring about reductions of photosynthetic activity in the mornings than was required to produce corresponding reductions in the afternoons. The afternoon determinations showed a reduction when approximately 75 % of the available soil moisture had been extracted, while morning determinations did not show a reduction until more than 90 % of the available soil moisture had been utilized.

Of the three investigations described above, only that of Schneider and Childers (10) includes a consideration of respiration. They found appreciable increases in the rates of respiration as wilting was approached. In the most severe state of wilting considered, the flaccid plant was found to evolve CO_2 at a rate 62 % greater than the turgid control plant.

A number of other investigations have included observations of photosynthesis in wilting plants, although not in as detailed a manner as the publications mentioned above. Verduin and Loomis (13) compared the rates of apparent photosynthesis (net CO_2 taken up in light) of turgid corn leaves with those of visibly wilted corn leaves. In 21 experiments wilted leaves averaged 37 % of the controls with values varying from 0 to 82 %. Thomas and Hill (11) noted that wilting reduced the photosynthetic activity of alfalfa.

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[3] Present address: Department of Agronomy, North Carolina State College, Raleigh, North Carolina.

Materials and Methods

The apparatus used for measuring CO_2 exchange and for controlling the environment of experimental plant material is shown in figure 1. The environmental control chamber (C) has an open-top metal box with a recess in the bottom to accommodate a three-liter can. A flange around the top served as a plate for holding a rubber gasket. The plexiglass top was sealed air-tight and connections were made through copper tubes fitted into the side of the chamber. This chamber was submerged with the top one inch below the water surface in a thermostatically controlled bath. A temperature of 25° ± 0.2° C was maintained within the chamber.

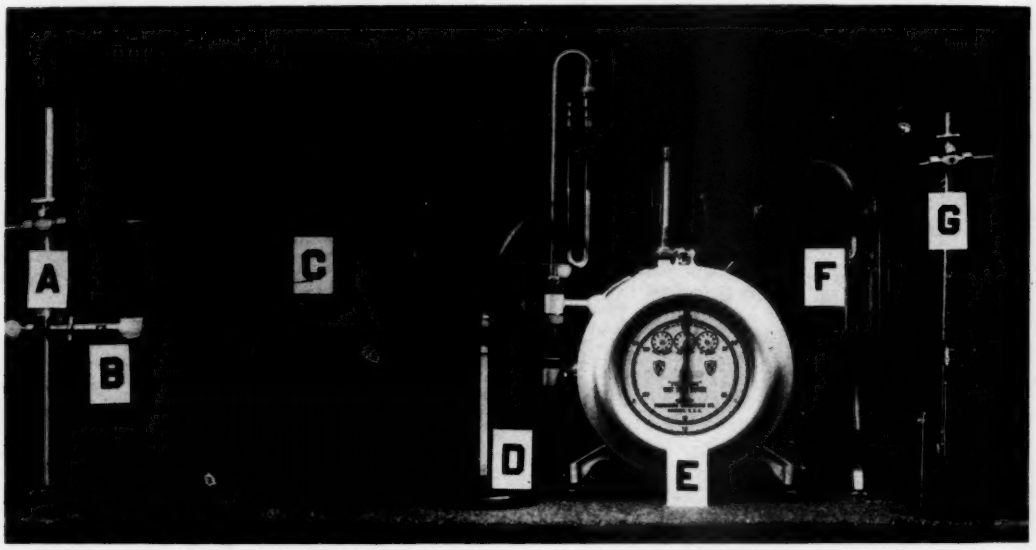


Fig. 1. Apparatus for controlling the environment of the experimental plants and for measuring CO_2 exchange. Components are as follows: A, needle valve for admitting air under pressure; B, pressure regulator; C, control chamber; D, flowmeter; E, gas meter; F, NaOH solution for absorbing CO_2; and G, needle valve for removing air by vacuum system.

Illumination of approximately 1,800 fc at 13 cm above the chamber floor was provided by a mixture of incandescent and white- and daylight-fluorescent lights mounted 20 inches above the chamber top.

The rate of air passage through the chamber was controlled by manipulation of needle valve (A) admitting air under pressure and needle valve (G) removing air by a vacuum system. Atmospheric pressure was maintained by a pressure regulator (B) on the incoming air line. The instantaneous rate of flow was indicated by a flowmeter (D) and accumulated amounts were indicated by a gas meter (E). A rate of approximately 190 liters of air per hour was used for all experiments.

The air stream was broken into eight, upwardly-directed openings in a tube located at the bottom and along the four sides of the chamber. Air was withdrawn through four holes in a tube which encircled the top of the chamber recess. The rate of air passage was so controlled that more than 99 % of the hourly rates were within ± 0.2 % of the mean hourly rate for the entire experimental period.

The total CO_2 of the air stream for one-hour periods was absorbed in a dilute standard solution of NaOH in a glass cylinder (F). Dispersion of the gas into the liquid was improved by the use of a sintered-glass tube and by using n-butyl alcohol in the absorbent at a concentration of 0.3 %. After an adsorption period, the excess standard base was titrated potentiometrically in a closed system. The CO_2 content of the air stream prior to entry into the chamber was established by blank determinations performed immediately preceding or following each one-hour experimental determination. The difference between the CO_2 contents of the influent and effluent gas streams indicated the amount of the net CO_2 exchange by the plant system in the experimental chamber. Variation among groups of four determinations, where no treatment effect was involved, was small. Less than 5 % of the individual determinations fell outside a range of 1.5 mg. The amount of net CO_2 exchange varied from 5 to 55 mg of CO_2 per hour for the various experimental treatments.

The CO_2 content of the air was not constant throughout each day but frequently showed a depression during the afternoon. This variation was reduced through the use of a reservoir with a capacity which was 1 to 2 times as large as the volume of air used experimentally during the afternoon. This sys-

tem reduced the variation in the CO_2 content of the incoming air for the one-hour periods to less than 1 % of the mean value over a 24-hour period.

The relative humidity in the chamber with an enclosed plant was 85 to 95 % during the light period and 55 to 65 % during the dark period. The relative humidity of the influent air stream was 10 to 15 % at 25° C.

The Yolo loam soil used had a moisture equivalent of 22.1 % and a permanent wilting percentage of 12.7 % as determined by the sunflower method. Procedures for these determinations have been published (7). Since a soil moisture-tension curve was not available for this soil, the data have been plotted as a function of time rather than as a function of soil moisture tension.

Five ladino clover plants were established from seed in each of several three-liter metal containers. These plants were grown and maintained under artificial light and controlled temperatures. At the beginning of the experiments the older leaves were removed, leaving only 9 to 12 of the younger leaves on each stolon.

A correction for the CO_2 produced by roots and soil microorganisms was accomplished by making an almost complete separation of the aerial and root environments. A metal lid with holes for each stolon was soldered to the top of the can. These holes were sealed with split cork stoppers and sealing compound. Air was passed slowly over the surface of the soil to provide root aeration using an arrangement which prevented CO_2 from escaping into the aerial portion of the chamber.

Where the net CO_2 taken up in light is measured, it is usually termed apparent photosynthesis since it represents the actual amount of CO_2 used in photosynthesis less the CO_2 produced by respiration. When the rates of respiration and apparent photosynthesis are added to give a corrected photosynthesis value, the assumption is tacitly made that rates of respiration in light and in dark are equal. Previous work by Weintraub (14) has indicated that this assumption can be essentially but not absolutely justified. Since it is such a useful assumption, it has been made for this work.

Results

Effects of Allowing Soil-moisture to Vary Between the Moisture Equivalent and the Permanent Wilting Percentage: Two experiments were conducted to study rates of respiration and photosynthesis as affected by soil-moisture contents between moisture equivalent and permanent wilting percentage. Plant containers were placed in the chamber while the soil-moisture contents were near the moisture equivalent. When the plants showed slight signs of moisture deficiency or when such signs were anticipated on the basis of time elapsed since the last irrigation, they were irrigated to the approximate moisture equivalent value. The plants were then allowed to extract the soil moisture to a point beyond the permanent wilting percentage. When the plants wilted distinctly they were again irrigated to the approximate moisture equivalent. Two days later they were removed from the chamber. Irrigations were applied only at the beginning of light periods. Throughout both experiments, determinations of the CO_2 exchanged over one-hour periods were made on the following schedule. During the twelve-hour light periods, four determinations were made within the last six hours, and during twelve-hour dark periods, four determinations were made within the last nine hours of darkness.

The influences of available moisture depletion on

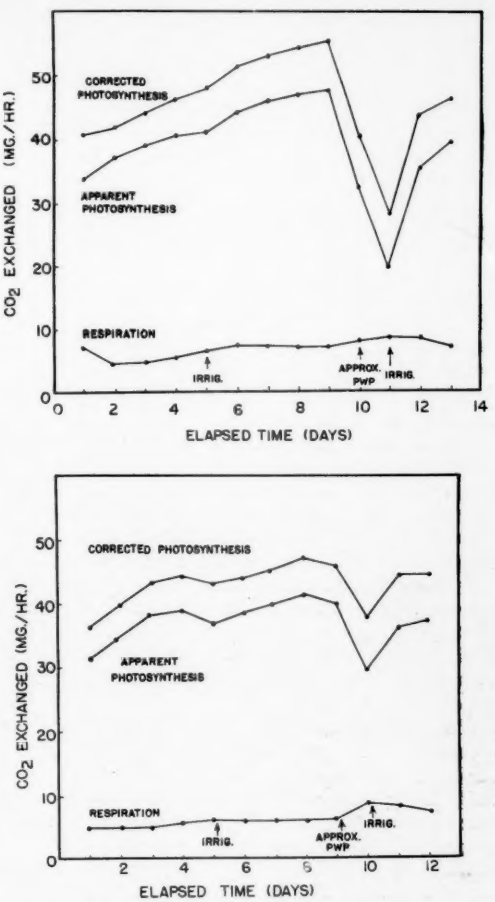


Fig. 2 (*above*). Effects of available moisture depletion on the average daily rates of photosynthesis and respiration in ladino clover. The points shown are the means of 4 determinations made during the last 6 hrs of a 12-hr light period. Irrigation on the 5th day preceded visible wilting.

Fig. 3 (*below*). Results of a study similar to that given in figure 2 except that irrigation on the 5th day followed appearance of wilting.

the daily rates of respiration and photosynthesis are shown in figures 2 and 3. These results suggest a tendency for the respiration rate of the ladino clover shoot to increase just before the permanent wilting percentage is reached. This increase, however, is much smaller than the accompanying change in photosynthesis rate. In preliminary experiments where the CO_2 produced by the soil-root complex was not removed from the air stream, this slight increase in respiration rate was not detected. Instead the combined respiration rate of clover shoots, roots, and soil microflora showed a decrease as the permanent wilting percentage was approached. It is evident from figures 2 and 3 that correction for shoot respiration has little effect on the interpretation of the photosynthesis curves. Thus the same conclusions can be drawn from either the corrected or the apparent photosynthesis curves.

In the experiment of figure 2, the enlarging leaf surface caused a gradual increase in the rates of CO_2 exchanged. On the fifth day the available moisture was nearly depleted, and the clover was irrigated. Although no wilting could be detected near exhaustion of the available moisture caused a slight dip in the photosynthetic rate. During the tenth day, wilting occurred accompanied by a marked reduction in photosynthetic rate. Wilting continued for an additional day before the plant was watered and allowed to recover. Such severe wilting caused many of the older leaves to die.

Soil-moisture conditions in the experiment represented by figure 2 were not identical to those prevailing in the second experiment given in figure 3. The irrigation applied on the fifth day in the first experiment preceded visible signs of wilting, while in the second experiment some wilting was evident prior to the corresponding irrigation. Loss of turgor was accompanied by a distinct drop in photosynthetic activity. Again during the ninth day the rate of photosynthesis diminished rapidly as distinct wilting characteristics of the permanent wilting percentages developed.

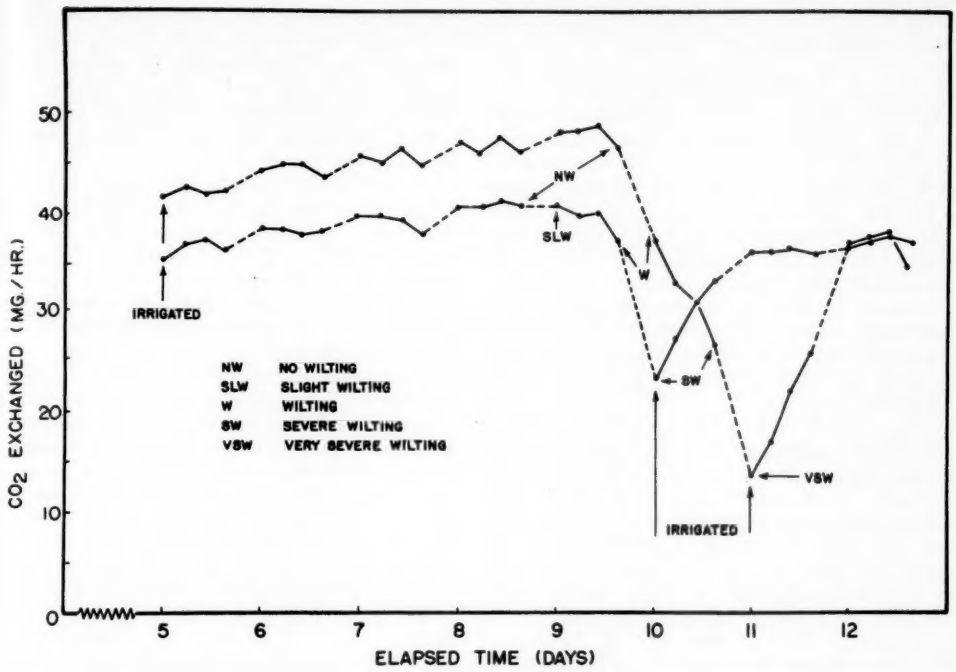
The effects of approaching wilting on photosynthetic rates are shown in greater detail in figure 4 for the latter portions of these same experiments. The points connected by the solid lines of the figure show the mean rates of CO_2 exchange during four one-hour periods occurring within the last six hours of successive light periods. In the experiment represented by the upper curve of figure 4, the photosynthetic rate was little affected by soil moisture depletion until the tenth day when wilting occurred during the first six hours of the light period. The CO_2 exchanged during the next four one-hour intervals diminished rapidly as the severity of wilting increased. Once the clover plant begins to wilt its rate of apparent photosynthesis appears to decline rapidly. On this assumption it is estimated that, as moisture becomes limiting, apparent photosynthetic activity drops to about 50 % of its maximum value in the course of 14 to 20 hours under the environment provided. When the plants were distinctly wilted, the apparent photosynthetic activity was about 70 to 80 % of its maximum value. At more advanced stages of wilting apparent photosynthesis was 40 to 50 % of the maximum, even though almost every petiole was collapsed and almost every leaf showing visible effects of reduced turgidity. Irrigation in this severe stage of wilting nearly doubled the rates of apparent photosynthesis within 6 hours. This increase was linear in nature with the total response to irrigation occurring within 24 hours. The original rate of apparent photosynthesis was not attained following irrigation. One reason for this may have been the loss of many older leaves caused by such severe wilting.

The occurrence of daily dark periods in these experiments hampers interpretation. In the experiment depicted by the upper curve of figure 4, wilting occurred at the beginning of the light period, while in the experiment represented by the lower curve, wilting began at the end of the light period. The advent of a dark period arrests the progressive changes in a wilting plant, thus producing some variability in the shape of the curves representing photosynthetic activity during the course of wilting. To avoid this problem, several experiments were carried out under continuous light.

Effects of Available Moisture Depletion under Continuous Light: Plants which had been under continuous light for over two weeks were placed in the chamber while the soil moisture was near the moisture equivalent. Two cycles from moisture equivalent to permanent wilting percentage were carried out. Carbon dioxide exchange measurements were made at frequent intervals as wilting was approached. Possible abnormalities in the growth and CO_2 exchange patterns of ladino clover under continuous light were not thoroughly investigated, but no visible abnormalities developed.

The results given in figure 5 are in substantial agreement with the previous experiments. No reduction in photosynthetic activity was noted until the approximate time of appearance of the first signs of wilting. As before, the photosynthetic activity declined rapidly with increased severity of wilting. Within approximately 20 hours after the first signs of wilting were detected, the rate of photosynthesis was reduced 42.5 % in the first experiment and 48.6 % in the second.

Effect of Irrigating When 80 % of the Soil Moisture Held between the Moisture Equivalent and the Permanent Wilting Percentage Had Been Extracted: The procedure for this experiment was the same as for the experiments whose results are presented in figures 2 and 3 except that irrigations were performed when an estimated 75 to 85 % of the moisture held between the moisture equivalent and permanent wilting percentage had been extracted. Irrigations were required three times during the course of the experiment. The soil-moisture content at the end of the 15-day experiment was within 10 %



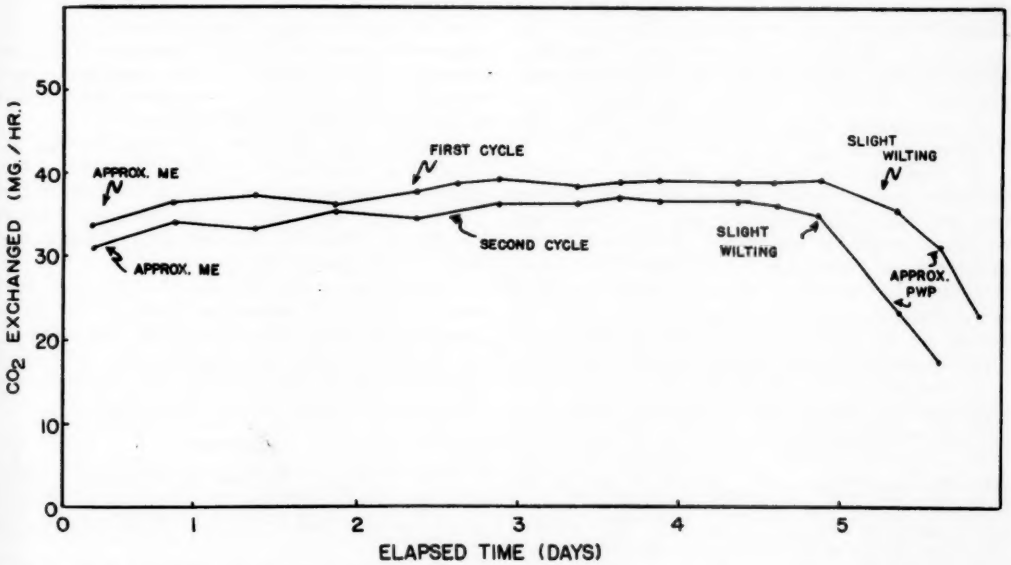


FIG. 4 (*above*). Effects of approaching wilting on photosynthetic rates. The points connected by solid lines show the mean rates of CO_2 exchange during 4 1-hr periods within the last 6 hrs of the 12-hr light periods. The remaining 18-hr periods are compressed and represented by the dotted portions of the curves. The upper curve represents in detail the latter portion of the experiment given in figure 2 and the lower curve that of figure 3.

FIG. 5 (*below*). Effect of available moisture depletion on the photosynthetic activity of ladino clover for two experiments carried out under continuous light. The points shown are the mean rates of CO_2 exchanged during 6-hr periods.

of the predicted value. No changes in the rates of apparent or corrected photosynthesis were noted as a result of these irrigations.

Discussion

The data from the several experiments reported suggest that in ladino clover the rate of photosynthesis (apparent or corrected) is affected little by removal of available moisture until the approximate time the first visible signs of wilting appear. Under the experimental conditions, these first signs of wilting have occurred when approximately 10 % of the total available moisture in the container remained to be extracted. In experiments where irrigations were delayed until at least 75 to 85 % of the available moisture had been extracted, no changes in the rate of photosynthesis were detected preceding or following irrigations.

The possibility that increasing leaf area during the course of the experiments could have been masked a gradual decrease in photosynthetic activity needs to be examined. Two lines of evidence indicate that such a masking was not apparent and that the conclusions presented above are, therefore, valid. If moisture had been limiting at stages earlier than those indicated, irrigations at these earlier stages should have produced an immediate increase in the rate of photosynthesis. Irrigations of these prescribed times did not cause an increase in photosynthesis. Measurements of the change in leaf area of ladino clover over a wide range of soil moisture tensions were made by the authors under conditions similar to those described for the experiments here. The rate of increase in leaf area was found to progressively decrease within the lower half of the available moisture range.

In applying the results of this study to other conditions, caution should be used since ladino clover may not exhibit the same response to soil-moisture variations under all conditions. For example, the occurrence and length of a dark period may be of importance in controlling the photosynthetic response of the plant to wilting. Also this study has been conducted, for the most part, using two or three moisture - equivalent to permanent - wilting - percentage cycles per experiment. While extraction of soil moisture almost to the permanent wilting percentage may have no immediate effect on the CO_2 exchange of the plant, it is possible that any partial loss of turgor may be of importance over a period of time by influencing such plant processes as meristematic growth or the hormone and enzyme systems. The possible long term effects would not have been demonstrated by the technique used in these experiments.

Richards and Wadleigh (9) have pointed out that the effect of soil-moisture variations on plant behavior depends in part on the type of plant used as an indicator and on the criterion of measurement. The differential effect of increasing moisture stress on various aspects of the physiological functioning of ladino clover has been reported (4). For ladino clover, the rate of petiole elongation progressively decreases within the lower half of the available moisture range, and ceases as the wilting point is approached (8). Thus the rate of petiole elongation, which is dependent upon cellular elongation, is much more sensitive to depletion of available moisture than is photosynthesis as indicated by the experiments reported in this paper.

It is of interest to raise the question whether the reductions in photosynthetic rates noted in the present study can account quantitatively for the decreases in dry weight production of ladino clover reported by Hagan and Peterson (3) to occur as the available moisture nears depletion. They found that allowing 75 % of the available moisture to be extracted before irrigation caused no significant reduction in dry matter yield of clover grown in containers but that delaying irrigation until just before the appearance of wilting reduced dry matter production approximately 30 %. In the present experiments, however, near exhaustion of the available moisture caused no detectable retardation of photosynthetic activity. The slightly increased rates of respiration reported here as wilting appeared, seem to be insufficient to account for the loss of dry matter production in Hagan and Peterson's experiments. Thus the possibility exists that gradual depletion of available soil moisture may affect some physiological processes upon which growth is dependent before the rate of photosynthesis is appreciably retarded. Allmendinger et al (1) reported that reduction in growth of apple trees as measured by terminal elongation of branches occurred when four-fifths of the available moisture was used, but this degree of extraction did not cause a reduction in rate of photosynthesis.

Schneider and Childers (10) and Bourdeau (2) found that photosynthesis by certain species of trees gradually decreased as soil moisture was reduced from the moisture equivalent to the permanent wilting percentage. Such results are quite different from those of the present study.

The results of Allmendinger et al (1) with apple trees, Loustalot (7) with pecan trees, and the present investigations with ladino clover all lead essentially to the same conclusions, namely; that the rate of photosynthesis is little affected until the permanent wilting percentage is closely approached.

Summary

The effects of variations in soil-moisture content over the available range on the rates of apparent photosynthesis and respiration in ladino clover are reported.

Rates of apparent photosynthesis were computed from the CO_2 exchanged as measured titrimetrically. The special apparatus used to control the plant environment during the experiments is described.

Plants irrigated when approximately 80 % of the soil-moisture held between the moisture equivalent and the permanent wilting percentage had been extracted showed no change in photosynthetic activity

preceding or following irrigations. No reduction in the rate of photosynthesis was detected as soil moisture was extracted, until the approximate time the first visible signs of wilting appeared. As the severity of wilting increased, the photosynthetic rate declined rapidly. When the plants were distinctly wilted, photosynthetic activity had been reduced to approximately 75 % of its maximum value. Severely wilted plants irrigated one day after the permanent wilting percentage had been reached showed 40 to 50 % increases in rate of photosynthesis within 6 hours. Experiments carried out under continuous light supported the above conclusion.

The respiration rate of the aerial portions of the ladino clover plant tended to increase slightly as wilting appeared. These increases were relatively much smaller than accompanying changes in photosynthesis.

Results of these studies are in accord with those of Allmendinger et al on apple trees and of Loustalot on pecan trees, but differ in degree from those of Schneider and Childers on apple trees.

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A STUDY OF THE ROLE OF THE HYDROGEN ION IN THE MECHANISM OF POTASSIUM ABSORPTION BY EXCISED BARLEY ROOTS[1]

T. R. NIELSEN and ROY OVERSTREET

Department of Soils, University of California, Davis and Berkeley, California

The hydrogen ion has an important place in many theories of cation accumulation by plants including those of Brooks (3), Osterhout (13), Briggs (2), Lundegårdh (10), and Jacobson, Overstreet, et al (9). All of these workers postulated that the absorption of cations takes place by means of an exchange of the cation for a hydrogen ion from the root, and this hypothesis is supported by experimental evidence. Perhaps the most convincing evidence on this point is the physiological acidity which develops when plant roots are placed in a salt solution, such as K_2SO_4, containing a rapidly accumulated cation and slowly accumulated anion (8).

Of the above-mentioned hypotheses, all except that of Jacobson, Overstreet, et al have in common the postulation that the driving force for accumulation of cations against a concentration gradient is a hydrogen ion concentration gradient in the opposite direction. Hoagland and Broyer (8) conducted experiments designed to ascertain whether such a gradient in hydrogen ion concentration was necessary for cation absorption. These workers concluded that cations could be accumulated against a gradient in their concentration even though the pH in the exter-

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[2] This paper is based in part on work performed under contract No. AT-(11-1)-34, project 5, with the Atomic Energy Commission.

nal solution was lower than that of the expressed root sap.

Overstreet and Jacobson (14) adopted the absorption model of Rosenberg in proposing, as a working hypothesis, the following mechanism for mineral absorption by plant roots. A membrane or region exists in the cytoplasm of root cells which is impermeable to free ions, but permeable to complexes formed by the reaction of ions with compounds designated as HR and R′OH. The compounds HR and R′OH are produced at the external interface of this region where they can combine with anions and cations forming complexes as follows:

$$\text{(a)}\quad M^+ + HR \rightleftarrows MR + H^+$$
$$\text{(b)}\quad A^- + R'OH \rightleftarrows R'A + OH^-$$

where M^+ represents a cation, A^- an anion.

The complexes MR and R′A then diffuse across this region and at the inner boundary are chemically altered in such a way that the R's can no longer serve as carriers, M^+ and A^- being secreted to the region beyond the inner boundary. An inward diffusion gradient of MR and R′A in the membrane region is maintained by the production of HR and R′OH at the external boundary and the alterations of MR and R′A at the internal boundary. Thus, the production and alteration of these compounds by metabolic chemical reactions is the driving force for the accumulation of ions against their concentration gradient, not the difference in hydrogen ion concentration between external solution and interior of the root.

The purpose of the present investigation was to obtain experimental data useful in testing the above hypothetical absorption mechanism.

Jacobson, Overstreet, et al (9) performed experiments designed to provide information on the reversibility of reaction (a), the cation-binding reaction. They placed excised barley roots in HCl solutions ranging in pH from 1 to 6.5 and measured the loss of K from the roots. However, the loss of K from the roots as a function of pH rather closely paralleled the loss of various other constituents such as inorganic phosphate, soluble organic phosphate, soluble nitrogen, etc. This casts doubt on the interpretation that the loss of K from the roots was merely a reversal of the K-binding reaction. In all probability, a more general disruption of the system occurred causing changes in permeability characteristics of the membranes and a resultant outward diffusion or leakage of cell constituents. This interpretation is supported by the fact that the dry weight of the roots decreased by approximately 25 % at a pH below 3. Also, roots that were treated with 0.005 *N* HCl solution exhibited a marked reduction in ability to absorb K from KCl solution subsequent to this treatment. These facts illustrate a basic difficulty in studying the effect of hydrogen ion on the absorption mechanism, namely, the difficulty in distinguishing between hydrogen ion effects on the actual absorption mechanism and other effects such as a general injury to the tissue. The possibility of injury to the root tissue has been excluded as rigorously as possible from data used to elucidate the reaction mechanism in the present study. Also, rates of ion accumulation have been investigated, rather than changes in the composition of the root material resulting from treatments of arbitrary duration, in order to rule out secondary effects and to provide data which can be interpreted in terms of the kinetics of the ion absorption mechanism.

Experimental Methods

The experimental material used was excised roots of *Hordeum vulgare*, var. Atlas 46, cultured and prepared as described by Jacobson et al (9), except that the H_2O_2 treatment was eliminated because it proved to be injurious to Atlas 46 barley. On the sixth day following the initial soaking of the seed, the roots were excised just below the seed, cut into segments 1 to 2 cm in length, rinsed thoroughly in distilled water, and centrifuged for 5 minutes at 670 RPM with 13 cm centrifugal radius to remove the water adhering to their surfaces. Samples of root material were then weighed out and placed in various salt solutions, generally 3 gms of roots in 3 liters of solution. Since a root to solution ratio of 1 gm/l was used, the salt concentration of the solutions remained essentially constant throughout the absorption period. None of the solutions were buffered as it was considered desirable to avoid indirect effects of buffering ions on absorption. All solutions were aerated and maintained at 26° C.

Ion absorption was determined by chemical analysis of the root material before and after it was placed in a solution and was expressed as meq ion/1000 gm fresh root material. All solutions were aerated and maintained at 26° C. Potassium was determined as the cobaltinitrite, Br by a modified version of Van der Meulen's iodometric method (17) and Na by use of a flame photometer. Values of absorption from a given solution using root samples from one batch of material or from batches of root material produced on different dates varied less than 5 %.

Results

Potassium absorption from KBr solutions at several concentrations at pH 4 and 6 was determined for periods of time ranging from ½ to 6 hours. A series of identical KBr solutions was prepared at each concentration and pH, and samples of root material were placed in each solution for the specified period of time. The pH 4 solutions were adjusted in pH with HBr. The amount of K absorbed in each case is related to time in figure 1 at pH 6 and 4, respectively. The rate of absorption at any point in time is, therefore, obtained by the slope of the curve in each case. All of the curves in figure 1 are linear between the ½- and 3-hour points indicating a constant rate of absorption. Data on Br absorption from KBr solutions in the pH range from 4 to 6 obtained in the same manner as those in figure 1 show that Br absorption also remains constant during the first 3-hr period except during the initial half hour.

Since the rate of K and Br absorption from KBr

solutions remains constant between ½ hour and 3 hours of contact, the effects of pH on absorption were determined after periods of 3 hours. In figure 2 the amounts of K and Br absorbed during a 3-hr period

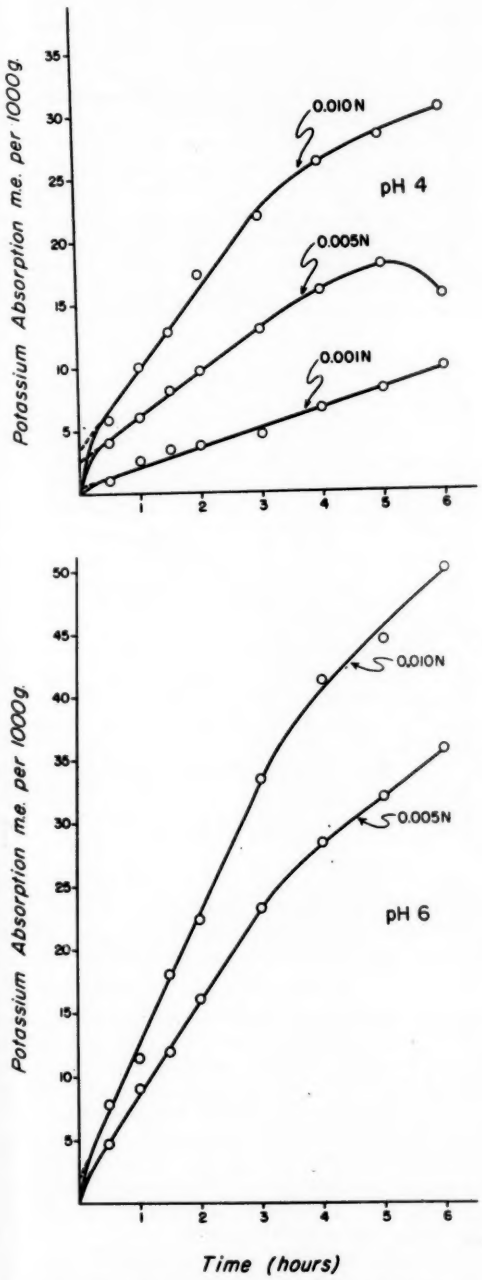


FIG. 1. Absorption of K from KBr solutions as a function of time at pH 4 (*above*) and pH 6 (*below*).

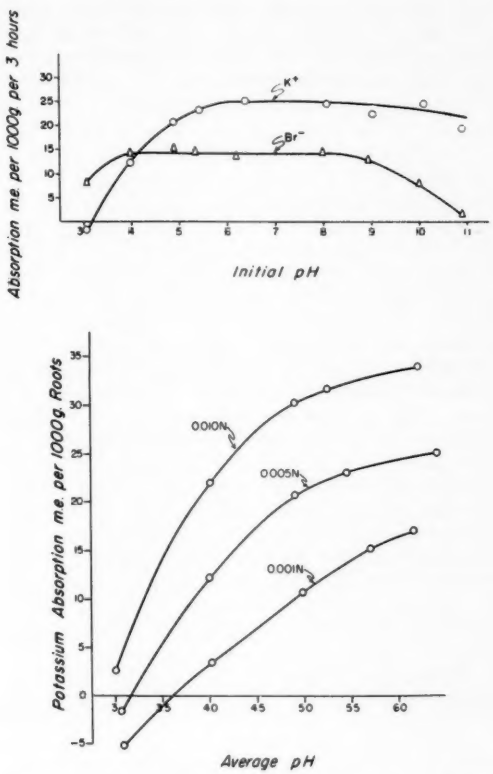


FIG. 2 (*above*). Absorption of K and Br from 0.005 *N* KBr solution as a function of pH.

FIG. 3 (*below*). Absorption of K as a function of pH from 0.001, 0.005 and 0.010 *N* KBr solutions; 3-hr absorption period.

from 0.005 *N* KBr are plotted as functions of the initial pH of the solutions. Solutions used for determination of K absorption had a constant K concentration at 0.005 *N*, and those used for the determination of Br absorption had a constant Br concentration also at 0.005 *N*.

In the above-mentioned solutions, the hydrogen ion concentration increased only slightly during the 3-hr absorption period in the case of the solutions more acid than pH 6.5. For example, the pH of the solution initially at 6.5 was 6.3 at the end of the 3-hr absorption period. However, in the case of the basic solutions, the changes in pH during the absorption period were much greater. The final pH of the solution initially at pH 10.1 was 7.5, for example. Hence, the absorption data of figure 2 for the solutions at pH higher than 6.5 must be considered only as approximations indicative of a trend.

Because of the large variations in pH in the higher pH range, primary attention was directed toward the pH range below pH 6.5. In figure 3, the amounts of K absorbed from 0.001, 0.005, and 0.010 *N*

KBr solutions is related to the average of the initial and final pH of these solutions. The pH of the solutions was adjusted with HBr. Negative values of absorption indicate loss of K from the root tissue.

To verify that the hydrogen ion was not per se injurious in the pH range from 4 to 6, barley root material was placed in dilute HBr solutions at pH 4, 4.5, and 6 for a 3-hr period, and subsequent to this treatment, K absorption from 0.005 *N* KBr was determined as a function of time. Twenty-five gm samples of root material were placed in 15 liters of each solution for a 3-hr period, removed, washed with distilled water, and re-centrifuged in the standard manner. The fresh weight and K content of this material was determined and 3-gm samples were weighed out, placed for varying time periods in 3-l volumes of 0.005 *N* KBr solution at pH 6, and analyzed. The K absorption data were similar to those of figure 1 in that the curves were curvo-linear up to the ½-hour point and linear beyond this point. However, the curves deviated from linearity beyond the 2-hr point which might be expected since at this point the root material had been in the pre-treatment solutions and the KBr solution for a total of 5 hours. The rates of K absorption were identical in the case of the root material pre-treated at pH 4.5 and 6, while the rate of K absorption by the root material pretreated at pH 4.0 was somewhat lower.

Earlier work with barley and other root tissue has demonstrated that Ca and other polyvalent cations

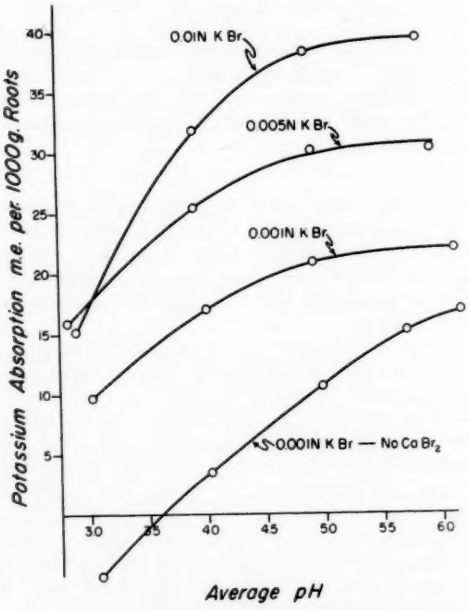
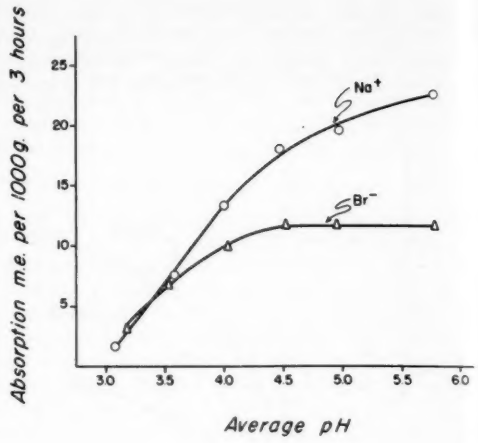


FIG. 4. Potassium absorption as a function of pH from KBr + 0.005 *N* $CaBr_2$. Also, K absorption from 0.001 *N* KBr without $CaBr_2$ as a function of pH; 3-hr absorption period.



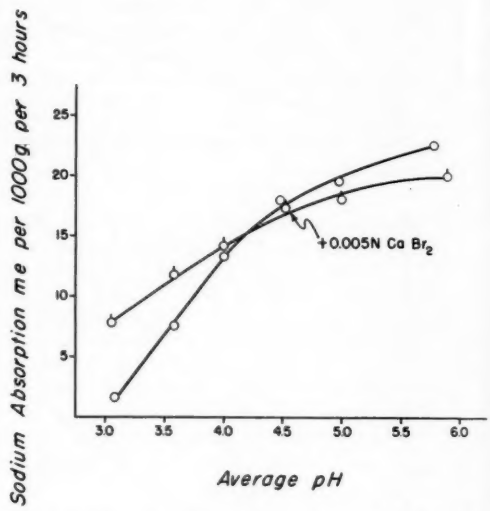


FIG. 5 (*above*). Absorption of Na and Br from 0.005 *N* NaBr as a function of pH.

FIG. 6 (*below*). Absorption of Na from 0.005 *N* NaBr and from 0.005 *N* NaBr + 0.005 *N* $CaBr_2$ as a function of pH.

have an ameliorating effect on injury to the tissue resulting from hydrogen ions (1, 6, 9). It was considered desirable, therefore, to investigate the hydrogen ion effect on K absorption in the presence of Ca ion. In figure 4, the amount of K absorbed in a 3-hr period from 0.001, 0.005 and 0.010 *N* KBr solutions all 0.005 *N* in $CaBr_2$ is plotted as a function of average pH of the solution. HBr was used to obtain the pH indicated. Data of K absorption from 0.001 *N* KBr alone is included for comparison.

To verify that rates of K absorption from mixtures of KBr and $CaBr_2$ are constant during the initial 3 hrs of contact, K absorption was determined as a function of time from the 0.005 *N* KBr + 0.005 *N*

$CaBr_2$ mixture at pH 6 and 4. This was done in the manner as indicated above for the experimental results presented in figure 1. The curves were linear throughout the initial 3-hr period indicating constant rates of absorption.

Sodium and Br absorption from 0.005 *N* NaBr solutions of varying pH were determined in order to compare the H-Na interaction with that of H-K. Also, Na absorption from 0.005 *N* NaBr + 0.005 *N* $CaBr_2$ mixtures at several pH's was determined. A series of 0.005 *N* NaBr and 0.005 *N* NaBr + 0.005 *N* $CaBr_2$ solutions were prepared and adjusted to varying pH with HBr. Samples of root material were placed in each solution for a 3-hr period and the Na and Br absorptions were determined. In figure 5, the Na and Br absorption from 0.005 *N* NaBr is plotted as a function of the pH of the solution. In figure 6, Na absorption from 0.005 *N* NaBr and from the 0.005 *N* $CaBr_2$ mixture is plotted as a function of the pH of the solution.

Discussion

Data have been presented showing the effect of hydrogen ion concentration of solution on K and Br absorption by barley roots. The interpretation of these data in terms of a direct hydrogen ion effect on the absorption mechanisms requires the establishment of a lower pH limit above which injurious effects of hydrogen ion can be excluded. One line of evidence useful for this purpose is the similarity in shape of the curves of figure 1. The curves at both pH 4 and pH 6 show a constant rate of K absorption between the ½- and 3-hr points. If hydrogen ion injury to the tissue were of any consequence at pH 4, one would expect a progressive decrease in rate of absorption as the root material remained in the solution for increasing periods of time. Since no such decrease in absorption rate occurs, in the case of either K or Br absorption, one must conclude either that hydrogen ion injury at pH 4 is unimportant and that the effect of the hydrogen ion concentration in decreasing the rate of K absorption is a direct effect on the K absorption mechanism, or that a peculiar sort of hydrogen ion injury is operative, namely, one that affects the root material almost instantaneously when roots are placed in a solution and has no further effect. The latter possibility is highly unlikely.

A further criterion of hydrogen ion injury is the effect of treatments of root material at various hydrogen ion concentrations on the subsequent ability of the material to absorb ions. The data showing rates of K absorption subsequent to treatments of root material at pH 4, 4.5, and 6 (mentioned above) indicated that both the pH 4.5 and 6 treatments had the same effect on subsequent K absorption. However, the subsequent rate of K absorption by the root tissue treated at pH 4 was somewhat lower. This indicates an injurious effect of hydrogen ion under the conditions of this experiment.

Other experiments with barley root tissue (6, 9) have indicated that the presence of salt in solution mitigates to a considerable extent the injurious effect of hydrogen ion. This must be taken into consideration in making inferences from the above data on hydrogen ion injury in the absence of salt applicable to those in the presence of KBr (figs 1, 2, and 3). Considering both of the above-mentioned criteria of hydrogen ion injury, one must conclude that hydrogen ion injury, can be ruled out in interpreting the data of figures 2 and 3, certainly between pH 4.5 and 6 and very probably between pH 4 and 6. Moreover, since the data in figure 1 demonstrate that the rates of K absorption remain constant during the initial 3 hrs of contact except for a slightly higher rate immediately after the roots are placed in the solution, the amounts of K absorbed given in figures 2 and 3 are measures of these constant rates of absorption. The same is true of the amounts of Br absorbed given in figure 2.

From figure 2, it is evident that in the range of pH from 4 to 6 the rates of Br absorption is independent of hydrogen ion concentration while the rate of K absorption decreases markedly as the hydrogen ion concentration increases. Since it has been established that hydrogen ion is not injurious in this pH range, the differential effect of hydrogen on K and Br absorption must be attributed to a direct effect of hydrogen ion concentration on the K absorption mechanism.

The data of figure 3 show that the magnitude of the reduction in rate of K absorption at a given hydrogen ion concentration varies with KBr concentration of the solution. For example, the rates of K absorption at pH 4 are 65.3, 53.4, and 20.0 % of the rates at pH 6 in the 0.010, 0.005, and 0.001 *N* KBr solutions, respectively.

Olsen (12) in his studies of ion absorption by intact rye plants, arrived at conclusions similar to those mentioned in the preceding two paragraphs concerning the influence of hydrogen ion concentration on K absorption. One important difference in behavior viz ion absorption between the intact rye plants used by Olsen and the root material used in this study is that absorption rates with the rye plants were reported to be independent of concentration at solution concentrations greater than 0.003 M, while in the case of excised barley roots the amounts of K absorbed increased with increasing concentration up to 0.075 M.

According to the ion absorption hypothesis of Jacobson et al (9), the initial step of the cation absorption mechanism is reaction (2), the cation-binding reaction. For the case of K absorption, the reaction would be

$$(c) \quad K^+ + HR \rightleftarrows KR + H^+$$

Moreover, since the methods used in this investigation for measuring ion absorption do not distinguish between ions bound as KR or R′Br and free ions liberated to the region beyond the membrane, the measured rates of absorption must be equal to the rates of the ion-binding reactions, assuming that the ion absorption hypothesis is correct. Overstreet et al

(15) concluded that the rate-determining factors in this reaction are the concentrations of K^+, HR, KR, and H^+ at the reaction site. The overall rate of reaction (c) would be the difference between the rate of the forward and reverse reactions. It would be expected, therefore, that increased concentrations of K^+ and HR at the site of the cation-binding reaction would increase the rate of K absorption; while increased KR and H^+ concentrations at this site would decrease this rate. If the assumption is made that the concentrations of K^+ and H^+ at the reaction site are direct functions of the bulk solution phase concentrations of these ions, the absorption rate should increase with increasing concentration of K and decrease with increasing concentration of hydrogen in the external solution.

The data of figure 3 are in agreement with the Jacobson-Overstreet hypothesis for the ion absorption mechanism and the assumption as stated above. Moreover, considering also the anion absorption hypothesis, one would expect that cation absorption would be depressed by an increase in hydrogen ion concentration and anion absorption by an increase in hydroxyl ion concentration. The differential effect on cation and anion absorption both in the lower and higher pH ranges is illustrated in figure 2.

The effect of Ca ion on the K-H ion interaction is illustrated in figure 4. The effect of Ca ion is to decrease considerably the magnitude of the depressing effect of the hydrogen ion on K absorption in the pH 4 to 6 range.

The increase in rate of K absorption caused by Ca (and other polyvalent ions (6, 16), the Viets effect (16)), is illustrated by the K absorption data from 0.001 *N* KBr and the 0.001 *N* KBr + 0.005 *N* $CaBr_2$ mixture (fig 4). These data also illustrate the marked pH-dependence of the Viets effect.

Overstreet et al (15) concluded that Ca ion in the solution increased the rate of K absorption in the concentration range where it stimulated K absorption because of its function as a cofactor in the utilization of the K complex KR. Thus, the concentration of KR at the site of the ion binding reaction, the external membrane interface, would be reduced in the presence of Ca ion, and consequently the rate of the reverse reaction in equation (c) would be decreased. The question which arises concerning the data of figure 4 is whether or not the role of Ca assumed by Overstreet et al will explain, at least qualitatively, the Ca ion effect on the H-K interaction.

With a reduced concentration of KR because of Ca ion, changes in the rate of the reverse reaction brought about by changes in hydrogen ion concentration would be expected to be reduced in magnitude since the rate of the reverse reaction depends on the concentrations of both KR and H^+. This would produce a smaller change in the rate of the over-all reaction, the difference in the rates of the forward and reverse reactions, as shown by figure 4. Hence, one can conclude that these data are consistent with the Overstreet et al hypothesis.

In interpreting the Na absorption data of figures 5 and 6 in terms of the Na absorption mechanism, one must assume that the 3-hr absorption values of Na absorption represent close approximations to the rate of absorption as in the case of K absorption. Two reasons for believing that the Na and K absorption processes are similar in the case of barley roots are 1) the amounts of each ion absorbed from NaBr and KBr solutions of equal concentration are similar, and 2) Jacobson et al (9) found marked competition in absorption between Na^+ and K^+ which they interpreted as indicating a single binding compound for these two ions. The curves of figure 5 for Na^+ and Br^- are quite similar to those of figure 2 for K^+ and Br^- absorption. Also, the Ca effect on the Na-H interaction is similar to that of the K-H interaction in that the depressing effect of hydrogen ion on the absorption of both these ions is decreased by Ca ion. However, the Ca ion effect on Na absorption at pH 6 is the opposite of the effect on K absorption, i.e., Ca increases the rate of K absorption and decreases the rate of Na absorption. This finding confirms unpublished data of Handley (7). An explanation for the differences between the Na-Ca and K-Ca interactions is not evident.

In the above discussion, two factors were considered of importance in the quantitative aspects of ion absorption 1) the ionic concentrations at the site of the cation binding reaction and 2) the ion absorption reactions themselves. The first consideration bears some relationships to the ion absorption hypothesis of what may be termed the Swedish school (4, 5, and 11).

This hypothesis apparently implies that the interaction of ions at the plant root surfaces is the only selective step of the ion absorption process, at least for the case of cation absorption. However, the hypothesis fails in the case of the Ca-K interaction of the data presented in figure 6. When Ca^{++} is added to a solution containing K^+, the amount of K^+ absorbed on the surface of plant roots in the solution would be expected to decrease, assuming that the cation adsorptive properties of plant root surfaces are similar to those of more fully understood absorbents such as clay particles or ion exchange resins. Hence, according to the hypothesis, the rate of K^+ absorption should decrease in the presence of Ca^{++}; yet actually it increases.

It is the view of the present authors that factors which affect the ionic distribution at plant root surfaces must be taken into account in the over-all ion accumulation process because of their effect on the ionic concentration at the site of the ion-binding reactions of the Jacobson-Overstreet hypothesis.

Summary

Data were presented showing that changes in hydrogen ion concentration of KBr solutions in the pH range from 4 to 6 had a marked effect on the rate of K absorption from these solutions by excised barley roots. Potassium absorption decreases with

decreasing pH. From measurements of K absorption as a function of time and other data, it was concluded that the effect of hydrogen ion on K absorption could be interpreted as a direct hydrogen ion effect on the K absorption mechanism. The observed hydrogen ion effects on K absorption were consistent with the ion absorption hypothesis of Jacobson and Overstreet.

The presence of Ca ion in solution was found to decrease considerably the depressing effect of hydrogen ion on K absorption. An explanation of this phenomenon in terms of the Jacobson-Overstreet hypothesis was presented.

Increasing hydrogen ion concentrations depressed Na absorption from NaBr solution in a similar manner as K absorption from KBr solution.

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CYTOCHROME OXIDASE CONTENT AND RESPIRATORY RATES OF ETIOLATED WHEAT AND BARLEY SEEDLINGS[1,2]

GEORGE FRITZ AND HARRY BEEVERS

Department of Biological Sciences, Purdue University, Lafayette, Indiana

Hill and Hartree (10) have reviewed the data which have accumulated during the last several years regarding cytochrome oxidase of plants, and the bulk of the evidence favors the view that this terminal oxidase plays an important role in respiratory oxygen consumption. Also Webster's recent survey (21) stressed the widespread distribution of this enzyme in dicotyledonous plants. Even so, there are several reports that the enzyme is missing from older cereal tissues. For example, Waygood (20) concluded that the oxidase is not present in extracts of wheat seedlings after the third day of development. Butler (4) reported inability to detect cytochrome oxidase in extracts from 5-day wheat roots, although Lundegårdh (16), using a specially designed spectrophotometer, was able to observe characteristic cytochrome absorption bands in intact roots of older wheat. Albaum and Eichel (1) demonstrated that the oxidase was present in extracts from oat seedlings one or two days old, but did not detect the enzyme in extracts from older seedlings (up to 6 days), and concluded that cytochrome oxidase operated only during the embryonic stages. James (12) detected cytochrome oxidase in barley embryos, but found no evidence for its exist-

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[2] This paper constitutes a portion of a thesis submitted by George Fritz to the Graduate School of Purdue University in partial fulfillment of the requirements for the Ph.D. degree. The work was supported by a grant from the Purdue Research Foundation.

ence in the tips of older barley roots (10 to 11 days). In a recent review article, James (13) summarized the information relating to terminal oxidases in barley roots and supported the hypothesis that a transition in respiratory agents takes place during maturation.

Although the finding that cytochrome oxidase is present in any particular tissue would not prove that it operates in respiratory oxygen absorption, nevertheless a demonstration of its presence in a tissue previously thought to be without the enzyme removes the most potent argument against the possibility of its participation. Thus it is of considerable interest that we were able, in a re-investigation of this problem, to detect the enzyme at all stages of development of etiolated wheat, barley, rice and oat seedlings. Having established that the enzyme was present, experiments were instituted in which the enzyme content of seedlings of different ages were evaluated. For comparison, parallel experiments were conducted with pea, in which Stafford (19) has shown that the cytochrome oxidase persists at least to the 6-day stage, the oldest seedling investigated. Enzyme assay values, when compared to the respiratory oxygen absorption of the living tissue, allowed a decision to be made as to whether sufficient cytochrome oxidase was present in the extracts to account for the oxygen absorption of the tissue from which they were derived. Experiments of this nature cannot by themselves exclude the possibility of participation of other terminal oxidases. However, any suggestion from experiments in vivo that cytochrome oxidase is an important terminal oxidase in a given tissue would seem to require for its support a demonstration that a sufficient amount of the enzyme is present to mediate a major portion of the respiratory oxygen absorption.

Materials and Methods

Preparation of Enzyme Extracts: Etiolated seedlings of Vigo wheat (*Triticum vulgare*), Kentucky No. 1 barley (*Hordeum vulgare*), Alaska pea (*Pisum sativum*), Victory oat (*Avena sativa*) and rice (*Oryza sativa*) were used as sources of cytochrome oxidase. The seeds were disinfected by soaking for 8 hours in a 1 % solution of Arasan SF (E. I. DuPont de Nemours and Company) to which a sprinkling of "Tide" had been added as a wetting agent. Pea seeds were planted in vermiculite (heat-expanded mica), and were watered daily with tap water; the cereal seeds were treated similarly, except that the supporting medium was sand. For a few preliminary experiments, plants were grown as described in (7).

The soaking and subsequent germination and growth took place at 29° C in a dark room with occasional red light. At harvest time, sand or vermiculite was washed away with running water, and a sample of seedlings (usually 50 to 100) was selected. The pea cotyledons and the cereal endosperms were removed and discarded, and the plants were separated into root and shoot as required. After drying with paper toweling and weighing, the tissue was cut into small pieces with scissors, and stored at about 10° C for one to two hours. The enzyme extract was prepared by grinding the tissue with half its weight of 0.1 M phosphate buffer (pH 7.7) in a previously chilled mortar and pestle; a small quantity of white sand was sprinkled on the tissue to facilitate grinding. The mash was recovered and strained through a piece of broadcloth, hand pressure being exerted to assist the extraction of plant juice, and the volume of the extract was recorded. The plant extract, containing the cytochrome oxidase, was used without delay and in the spectrophotometric experiments it was maintained at 0° C until it was added to the cuvettes.

The pH of the extract was 6.9 to 7.3, depending upon the kind and age of the tissue. For spectrophotometric experiments, no adjustment of pH was made, since the volume of plant juice added to a cuvette was very small in comparison to the volume of reduced cytochrome c solution which was buffered at pH 7.2. For manometric experiments, the extract was adjusted to pH 7.4 except for certain experiments with pea juice, as noted, when the pH was adjusted to 7.0.

Rate of Respiration: The rates of oxygen absorption of intact seedlings, or of the separated roots and shoots, were measured manometrically at 25° C. The tissue was cut into small pieces with a razor blade and placed into Warburg flasks containing water; KOH-saturated paper was placed in the center well. Seedlings selected for measurement of respiratory rates were of average size, and the recorded rates of oxygen absorption represent the means of several determinations.

Manometric Technique: The manometric method of measuring cytochrome oxidase activity involves the addition of cytochrome c and a reducing agent to the enzyme preparation, the function of the reductant being to reduce the cytochrome c non-enzymatically, after it is oxidized by the oxidase (18). The reducing agent used in most of the experiments was *p*-phenylenediamine (PPD) and for each experiment a solution (0.33 M adjusted to pH 7.4) was prepared a few minutes before use.

Measurement of oxygen consumption was made in standard Warburg respirometers at 25° C; the gas phase was air. The flasks contained enzyme extract (1.0 ml for wheat and barley and 0.5 ml for pea), cytochrome c solution (made by dissolving 2.0 mg "Sigma" cytochrome c per ml water), reducing agent (0.75 ml in the side-arm) and deionized water to a volume of 2.5 ml. Each flask contained KOH saturated paper in the center well. After tipping, readings were taken at 5-minute intervals for 15 minutes. The initial rate of oxygen uptake was estimated graphically, and converted to the rate per hour after subtracting the value for autoxidation of the reducing agent; these rates were a measure of activity at any given concentration of cytochrome c.

The rate of autoxidation of the reducing agent was determined by measuring its rate of oxygen uptake in the presence of a constant concentration of cytochrome c, at several concentrations of enzyme, and

extrapolating to zero enzyme concentration, according to the method outlined by Schneider and Potter (17) and Slater (18). The rate of autoxidation of PPD per Warburg flask was small (20 μl O_2/hour at the highest cytochrome c level employed) in comparison with the rate of enzymatic oxidation (from 150 to 600 μl O_2/hour).

SPECTROPHOTOMETRIC TECHNIQUE: The spectrophotometric method of determining cytochrome oxidase activity, introduced by Hogness (11) is based upon the direct enzymatic oxidation of reduced cytochrome c by oxygen, and the rate of oxidation is measured by the rate of decrease of optical density at 550 mμ, where the alpha-band of reduced cytochrome c exhibits a peak. It should be stressed that the spectrophotometric method of measuring cytochrome oxidase is much more sensitive than the manometric method. Thus, a readily measurable decrease in optical density of 0.02 divisions per *minute* during the oxidation of reduced cytochrome c corresponds to the absorption of approximately one μl oxygen per *hour*, an uptake which is scarcely detectable manometrically.

The procedure was based upon that described by Cooperstein and Lazarow (5). Cytochrome c was dissolved in 0.03 M phosphate buffer (pH 7.7) so that there was 0.66 mg cytochrome c per ml, and reduction was accomplished by adding sufficient solid sodium hyposulfite ($Na_2S_2O_4$) to give a concentration of 0.004 M. A fine air stream was passed through the solution for 3 to 5 minutes to oxidize excess hyposulfite. (After aeration, the pH of the solution was 7.2). Reduced cytochrome solutions were prepared in small quantities as needed, and reduction was effected immediately before use.

Measurements of absorbance at 550 mμ were made at room temperature (21 to 25° C) with a Beckman Model B spectrophotometer. The absorbance was recorded 15 seconds after adding the plant extract (usually 0.02 ml) to 3.0 ml of a cytochrome c solution and readings were taken at half-minute intervals. Linear curves were obtained for 1.5 to 2 minutes and optical density change per minute was the measure of activity at any given concentration of cytochrome c. The change in optical density was converted to μl oxygen absorbed, by the procedure outlined below. Sufficient oxygen is present in solution so that it does not limit the rate of the reaction and autoxidation of the reduced cytochrome c during the 2-minute reaction period was negligible.

No change was ever detected upon the addition of catalase (Bios Laboratories, Inc., New York) in the ability of each enzyme extract—prepared from either root or shoot systems of wheat, pea or barley seedlings—to catalyze the oxidation of reduced cytochrome c, when tested spectrophotometrically. Consequently, the possibility of peroxidase participation in cytochrome c oxidation was rendered unlikely.

EXPRESSION OF RESULTS: The procedure developed by Slater (18) was followed in assaying for cytochrome oxidase content and results are expressed in terms of catalytic ability of the enzyme at infinite cytochrome c concentration. To obtain these assay values, enzyme activities were determined at several levels of cytochrome c, and the activity at infinite concentration of cytochrome c was estimated by the extrapolation procedure of Lineweaver and Burk (15). For the manometric method, the reciprocal of the rate of oxygen absorption plotted against the reciprocal of the concentration of added cytochrome c; the rate of oxygen absorption in μl per hour at infinite concentration of cytochrome c was estimated by extrapolation, and was expressed as V_{max} per ml enzyme extract. Since the number of seedlings used and the volume of the extract were known the cytochrome oxidase content of a single seedling could then be derived from this figure.

In the spectrophotometric method (used when the seedlings were separated into root and shoot), the optical density change (ΔD) per minute per 0.02 ml enzyme extract was determined for several levels of cytochrome c. As above, the reciprocals of these functions were plotted, and the optical density change (ΔD) per minute at infinite concentration of cytochrome c was estimated by extrapolation. This value was then converted into V_{max} per ml extract by application of equation (*1*):

(*1*) ΔD/min for 0.02 ml extract $\times$ 2500
$= \mu l\ O_2$ absorbed/hour per ml extract
$= V_{max}$ per ml enzyme extract

Then, as in the manometric method, the value for the cytochrome oxidase content of the root or shoot system (V_{max} per root or shoot system) was readily computed.

Equation (*1*) was derived from the fundamental relationship described by previous workers (2, 5, 9):

(*2*) concentration of oxidized cytochrome c
$$= \frac{D_r - D}{1.96 \times 10^4} \text{ moles per liter}$$

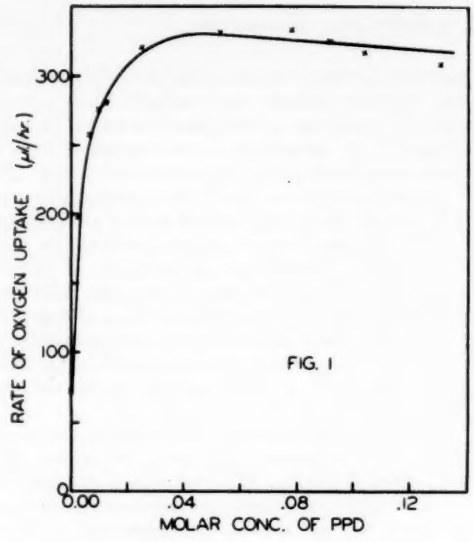
where D_r is the optical density reading after complete reduction with sodium hyposulfite, and D is the reading at any other time. The difference between the extinction coefficients for reduced and oxidized cytochrome c, at 550 mμ, is 1.96×10^4.

From equation (*2*) the following expression can be obtained.

(*3*) Oxygen utilized in μl/hr $\times$ ml enzyme extract
$$= \frac{\Delta D/\text{min for 0.02 ml extract} \times 50 \times 3 \times 10^{-3} \times 60 \times 22.4 \times 10^6 \times 0.25}{1.96 \times 10^4},$$

where 50 = conversion of 0.02 ml enzyme extract to 1.0 ml extract
3×10^{-3} = volume of reaction solution in liters
60 = conversion of minutes to hours
22.4×10^6 = conversion of moles of O_2 to μl of O_2
0.25 = equivalence in moles between cytochrome c and oxygen.

Equation (*3*) can be simplified to equation (*1*).


RATE OF OXYGEN UPTAKE (μl/hr)
300
200
100
0
0.00
.04
.08
.12
MOLAR CONC. OF PPD
FIG. 1

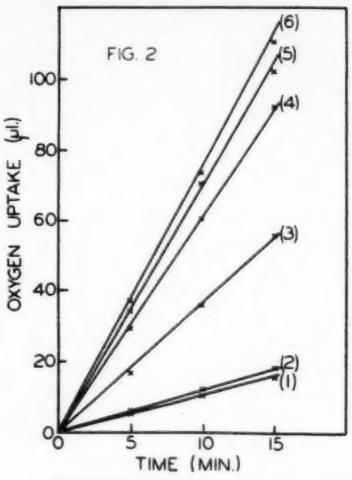
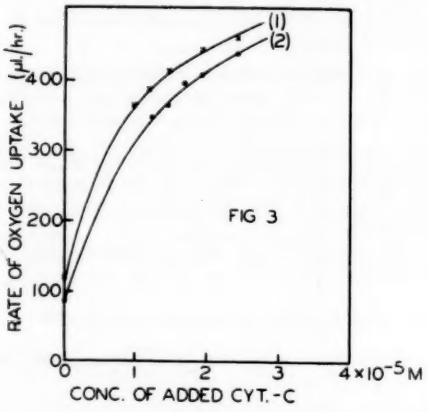
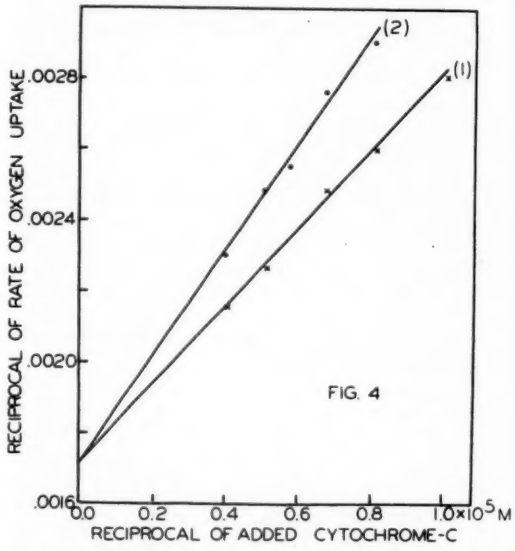
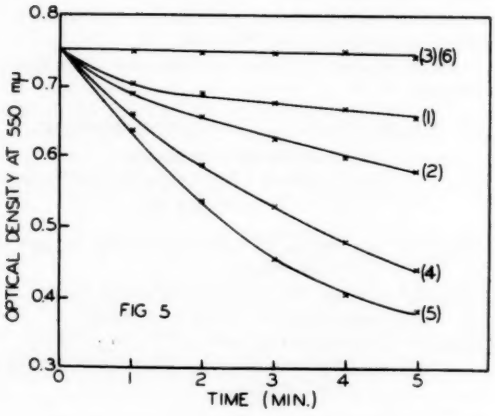


FIG. 2
OXYGEN UPTAKE (μl.)
100
80
60
40
20
0
0
5
10
15
TIME (MIN.)
(6)
(5)
(4)
(3)
(2)
(1)


RATE OF OXYGEN UPTAKE (μl/hr.)
400
300
200
100
0
0
1
2
3
4×10⁻⁵M
CONC. OF ADDED CYT.-C
FIG 3
(1)
(2)


RECIPROCAL OF RATE OF OXYGEN UPTAKE
.0028
.0024
.0020
.0016
0.0
0.2
0.4
0.6
0.8
1.0×10⁻⁵M
RECIPROCAL OF ADDED CYTOCHROME-C
FIG. 4
(2)
(1)


OPTICAL DENSITY AT 550 mμ
0.8
0.7
0.6
0.5
0.4
0.3
0
1
2
3
4
5
TIME (MIN.)
FIG 5
(3)(6)
(1)
(2)
(4)
(5)

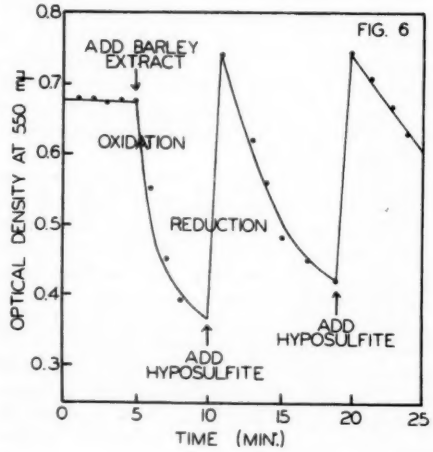


FIG. 6
OPTICAL DENSITY AT 550 mμ
0.8
0.7
0.6
0.5
0.4
0.3
ADD BARLEY EXTRACT
OXIDATION
REDUCTION
ADD HYPOSULFITE
ADD HYPOSULFITE
0
5
10
15
20
25
TIME (MIN.)

Results

Factors Affecting Activity: A series of manometric studies was performed to ascertain the effect of various factors upon the activity of cytochrome oxidase, and to clarify the best conditions for extraction and the requirements for measurement of maximal activity. For this purpose, extracts from 4-day wheat seedlings served as the source of enzyme.

The addition of PPD to the wheat extract produced a marked increase in the rate of oxygen absorption (fig 2, curve 3). Reasons are given in the discussion for believing that this catalysis of PPD oxidation is ascribable to cytochrome oxidase and the endogenous cytochrome c of the extracts. The addition of cytochrome c to the extract caused no increase in the rate of oxygen absorption; however, when cytochrome c was added to a mixture of the extract and PPD, a sharp stimulation in the rate of oxygen absorption was brought about (fig 2).

The effect of increasing the concentration of PPD upon the rate of its oxidation by the wheat extract in the presence of a fixed amount of added cytochrome c is shown in figure 1. It is evident that low concentrations of reducing agent limit the rate of the reaction, and to obtain maximal rates of oxygen uptake, it is necessary to employ a saturating level of the reductant. Accordingly, a final concentration of 0.1 M PPD in the Warburg flask was used in all later experiments.

Figure 2 shows that the rate of oxygen uptake (which was shown to be thermolabile) also depended upon the amount of cytochrome c which was added. The endogenous oxygen absorption (curve 1) was virtually unaffected by the addition of cytochrome c alone (curve 2). Curve 3 shows the oxygen absorption in the presence of a saturating amount of PPD. The rate of oxidation was increased (curves 4 to 6) by the addition of cytochrome c to the mixture of PPD and extract, and this stimulation demonstrates the existence of cytochrome oxidase in the extract. It is clear from figures 3 and 4 that higher rates of O_2 uptake can be induced as the concentration of cytochrome c is raised above 2.5×10^{-5} M and that, in fact, cytochrome c ceases to be a limiting factor only when its concentration is infinitely high. As a result of this, measurements of cytochrome oxidase activity at a cytochrome c level of 10^{-5} M give values which are only slightly greater than half of those possible at an infinitely high concentration of substrate, and at 2.5×10^{-5} M cytochrome c, the highest level employed, the observed rates are still less than 85 % of the maximum ones.

The effect of several other variables was also investigated. Increasing the phosphate concentration in the reaction vessel retarded the enzymatic oxidation of PPD. However, absence of phosphate, a condition obtained by using water as a grinding medium, resulted in extracts in which PPD was oxidized at a lower rate than when the enzyme was extracted in 0.1 M phosphate buffer. Also it was found that within rather wide limits on the alkaline side, the pH of the grinding medium had little effect upon the subsequent activity of the cytochrome oxidase; thus, equally active cytochrome oxidase extracts could be prepared from grinding media between pH 7 and pH 10. But if the pH of the extracting medium was more acid than pH 7, the activity of the oxidase was markedly reduced. It appears that if certain limits of acidity are exceeded during the extraction process, maximum values of oxidase activity will not be obtained. The general procedure adopted was to grind the plant tissue in 0.1 M phosphate buffer (pH 7.7), the volume of buffer being equal to half the fresh weight of the tissue. The extracted juice (the pH of which was approximately 7.0) was adjusted to pH 7.4, since this was found to be the optimum pH for the enzymatic oxidation of PPD.

Choice of Reducing Agent: Slater (18), in his manometric study of the cytochrome oxidase of heart-muscle preparations, showed that the activity of cytochrome oxidase, measured by extrapolation to infinite concentration of cytochrome c, was the same when ascorbic acid was used as the reducing agent, as when PPD was used. Comparable results have now been obtained when extracts from 3-day pea seedlings were used as the source of oxidase, and when

Fig. 1. Effect of *p*-phenylenediamine concentration on the rate of its oxidation by 1.0 ml extract from 4-day wheat seedlings in the presence of 1.21×10^{-5} M added cytochrome c.

Fig. 2. Oxidation of *p*-phenylenediamine in the presence of 1.0 ml extract from 4-day wheat seedlings and increasing amounts of added cytochrome c. Concentrations of added cytochrome c and PPD: Curve 1—no cyt c, no PPD. Curve 2—2.42×10^{-5} M cyt c, no PPD. Curve 3—no cyt c, 0.1 M PPD. Curve 4—0.97×10^{-5} cyt c, 0.1 M PPD. Curve 5—1.45×10^{-5} M cyt c, 0.1 M PPD. Curve 6—2.42×10^{-5} M cyt c, 0.1 M PPD.

Fig. 3. Effect of concentration of added cytochrome c on the rates of oxidation of *p*-phenylenediamine and hydroquinone by 0.5 ml extracts from 3-day pea seedlings. Curve 1—0.1 M *p*-phenylenediamine; curve 2—0.12 M hydroquinone.

Fig. 4. Data of fig 3, plotted according to the procedure of Lineweaver and Burk (15).

Fig. 5. The oxidation of reduced cytochrome c by extracts from 11-day barley roots and shoots. The enzyme extract was added at zero time and the optical density was recorded at 550 mμ. Curve 1—0.03 ml root extract. Curve 2—0.12 ml root extract. Curve 3—0.12 ml boiled root extract. Curve 4—0.03 ml shoot extract. Curve 5—0.12 ml shoot extract. Curve 6—0.12 ml boiled shoot extract.

Fig. 6. The oxidation of reduced cytochrome c by cytochrome oxidase from 10-day barley roots. Root extract (0.3 ml) was added to reduced cytochrome c solution at 5 min, and a few crystals of sodium hyposulfite were added at the times indicated.

PPD and HQ were the reductants. Ascorbic acid was not used in the present studies, since the rate of oxygen absorption in the presence of pea, wheat and barley extracts which contain ascorbic oxidase, was excessively rapid, and subsequent addition of cytochrome c resulted in only a small stimulation of oxygen uptake.

The rates of oxidation of saturating concentrations of PPD and HQ, in the presence of 0.5 ml of extract from 3-day pea seedlings and increasing quantities of added cytochrome c, are shown in figure 3. The concentration of HQ required to produce maximal rates of oxygen absorption was 0.12 M. Since it was shown (6, 14) that autoxidation of HQ is very small at pH 7.0, but increases rapidly at higher pH values, the data for this experiment were obtained at pH 7.0, whether PPD or HQ was the reductant. Autoxidation of HQ was estimated by the method used with PPD, and found to be small (25 μl O_2 per hour per Warburg flask).

The rate of oxygen absorption, at the concentrations of cytochrome c used, was dependent upon the reducing agent employed, higher rates being obtained with PPD than with HQ. However, extrapolation of the data of figure 3 by the procedure of Lineweaver and Burk (15) showed that the activity becomes independent of the reducing agent employed at infinite cytochrome c concentration (fig 4). These results demonstrate that if enzyme activity is measured at low concentrations of cytochrome c, values obtained will be dependent upon the particular reducing agent employed. But at infinite cytochrome c concentration, this dependence upon reducing agent vanishes. Therefore, to evaluate the full activity of the enzyme, it is necessary to extrapolate to infinite cytochrome c concentration, a thesis already advanced by Slater (18); under these conditions, the assay values are the same when PPD or HQ is used.

Cytochrome Oxidase from 11-Day Wheat and Barley: In view of the reports regarding the disappearance of cytochrome oxidase from the tissues of cereal seedlings in post-embryonic stages (1, 12, 13, 20), it is of interest that we were able to demonstrate the presence of the enzyme in extracts from the roots, as well as from the shoots, of etiolated wheat and barley seedlings as old as 12 days, by both the manometric and the spectrophotometric methods. (In less exhaustive studies with extracts prepared from the whole seedling of etiolated oat and rice, cytochrome oxidase was readily demonstrated by the manometric method at all ages up to two weeks.) The results of manometric tests for cytochrome oxidase in root and shoot extracts from 11-day barley and wheat seedlings are shown in table I. When cytochrome c was added to the mixture of PPD and root extract, a small but definite increase in the rate of oxygen uptake was obtained, and demonstrated the presence of cytochrome oxidase in these extracts; on the other hand, when the shoot extracts were employed, the corresponding rates were almost doubled. Further additions of cytochrome c caused no further increases in oxygen absorption when PPD was added to the root extract. Since the manometric assay for cytochrome oxidase depends upon the ability of added cytochrome c to cause progressive increases in the rate of oxygen uptake, and the extrapolation of this data to infinite cytochrome c concentration, it is apparent that the small increases obtained with the root extracts do not lend themselves to this treatment and would in fact involve considerable error. Thus the manometric method is not suited for the assay of the enzyme in the root extracts.

Table I

Manometric Demonstration of Cytochrome Oxidase in Extracts from the Roots and Shoots of 11-Day Barley and Wheat Seedlings

	μL O_2 absorbed/hr	
	Barley	Wheat
Root extract	10	6
" " + cytochrome c	10	6
" " + PPD	164	144
" " + cytochrome c + PPD	192	174
Shoot extract	12	16
" " + cytochrome c	12	16
" " + PPD	132	308
" " + cytochrome c + PPD	239	592

The reaction mixture was at pH 7.4; 1.0 ml of extract was used; PPD concentration was 0.1 M and the added cytochrome c conc was 2.42×10^{-5} M.

On the other hand, the ability of extracts prepared from roots and shoots of 11-day barley and wheat seedlings to catalyze the oxidation of reduced cytochrome c solutions could be readily measured with the spectrophotometer (the results for barley are shown in figure 5). On a volume basis, the extracts prepared from shoots were found to be more active than those from the roots, as was found in the manometric method. The successive oxidation of reduced cytochrome c by the enzyme and its reduction by added crystals of sodium hyposulfite is shown in figure 6; if an excess of crystals is avoided, the cycle of oxidation and reduction can be repeated several times. Since the rates of oxidation of several concentrations of reduced cytochrome c could be readily determined by this method, and the data could be extrapolated to infinite concentration of cytochrome c, the spectrophotometric method was used in later work for the determination of cytochrome oxidase values of various tissue extracts, and served to confirm earlier results obtained by the manometric method.

Cytochrome Oxidase Assay Results: The determination of the cytochrome oxidase values of etiolated wheat, barley and pea seedlings, in terms of catalytic ability at infinite concentration of cytochrome c (V_{max}), was made by manometric technique for seedlings at several ages of development, and these results are tabulated in table II, together with the respiratory rates of the living seedlings. In table III are shown the corresponding spectrophotometric

data, but in this case, the root and shoot tissue was analyzed separately. The studies were terminated at the 12-day stage, when the etiolated seedlings had reached or passed their period of most active growth, as measured by size and weight and rate of respiration.

An obvious feature of the data of table II is that the assay values for pea, whether expressed as V_{max} per seedling or otherwise, are consistently higher than the corresponding values for wheat and barley. In addition, columns 2 and 3 show that even though the amount of cytochrome oxidase in a unit volume of extract and in a gram fresh weight of tissue decreases as the seedlings mature, the absolute amount of enzyme per seedling (column 4) actually increases during the first week of growth. Thereafter, the amount of enzyme per seedling is more or less maintained (wheat and barley) or is still further increased (pea). In this connection, it is interesting to note that the respiratory rate of a wheat or barley seedling does not increase significantly after the fourth day (column 5). In the case of pea, where the respiration rate continued to rise throughout the 11-day period, it may be that production of protoplasmic material is continuing during this time, and that this is reflected in the paralled increase in cytochrome oxidase.

It is apparent from comparing values in columns 4 and 5 (table II) that the assay values for the cytochrome oxidase content of a single seedling, in all these plants, are higher than the rates of respiration of seedlings of the same age; in the case of pea, these values are several times higher. (To the extent that our methods are comparable with those of Stafford (19), the results may be regarded as extending to at least 12 days the period over which cytochrome oxidase in excess of respiratory needs can be obtained from etiolated pea seedlings). Thus it would appear that there is more than sufficient cytochrome oxidase present in a single seedling of these plants to meet the respiratory oxygen requirements. However, the further investigation of the separated root and shoot systems (by the spectrophotometric method) revealed the important fact that the relationship between cytochrome oxidase value and respiratory rate is not the same in the two tissues (table III). For the shoot system, the values for V_{max} (column 2) are in all cases considerably higher than the corresponding values for respiratory rates (column 3). But for the root systems of wheat and barley, the values for cytochrome oxidase (column 4) are consistently lower than the corresponding respiratory rates (column 5); on the other hand, the reverse is true for pea roots. It is therefore clear that whereas the shoot systems of all three kinds of plants, and the root system of pea, contain amounts of cytochrome oxidase which are more than adequate to account for the O_2 absorption in respiration, the root systems of wheat and barley are apparently deficient in this regard. Also, it is evident that the cytochrome oxidase values for wheat and barley shoots were sufficiently in excess of the values for respiratory rates, that they masked the deficiency of the enzyme in the roots when the entire

Table II

Manometric Assay Values of Cytochrome Oxidase Content (V_{max}) and the Rates of Respiration of Single Seedlings of Various Ages

Age in days	V_{max}**/ml enzyme extract	V_{max}/gm fresh wt	V_{max}/seedling	Resp rate of seedling *
(1)	(2)	(3)	(4)	(5)
		Wheat		
2.2	632	712	15	14
3.0	555	625	35	24
4.2	513	478	43	30
5.4	490	470	54	31
6.1	490	466	58	35
7.4	486	445	54	32
9.1	414	390	50	29
11.0	384	328	42	31
		Barley		
4.1	390	424	39	32
6.2	329	346	48	36
8.1	303	327	53	39
10.5	266	289	47	33
12.1	259	270	43	31
		Pea		
3.8	1000	1015	260	51
5.0	870	859	261	72
6.9	770	852	600	97
8.3	714	748	606	127
9.3	690	786	897	180
11.1	523	616	1104	190

* Respiratory rate is expressed in μl O_2/hr.

** V_{max} is a measure of the cytochrome oxidase activity of the tissue in terms of μl O_2/hr at infinite cytochrome c conc (see text for details).

Table III

Spectrophotometric Assay Values of Cytochrome Oxidase Content (V_{max}) and the Rates of Respiration of the Root and Shoot Systems of Single Seedlings of Various Ages

Age in days	Shoot system		Root system		Sum of V_{max} per shoot + root systems
	V_{max}**	Resp rate *	V_{max}	Resp rate	
(1)	(2)	(3)	(4)	(5)	(6)
		Wheat			
4	49	13	6	12	55
8	72	21	5	17	77
12	53	15	8	10	61
		Barley			
4	36	14	4	15	40
8	31	25	3	19	34
12	40	18	2	13	42
		Pea			
4	110	15	120	27	230
8	400	36	330	45	730
12	820	127	190	65	1030

* Respiratory rate is expressed in μl O_2/hr.

** V_{max} is a measure of the cytochrome oxidase activity of the tissue in terms of μl O_2/hr at infinite cytochrome c conc (see text for details).

seedling (including root and shoot systems) was analyzed.

By summing the separate contributions of the root and shoot systems, the cytochrome oxidase values for the entire seedling were obtained (table III, column 6). These spectrophotometric values can be compared with those achieved by the manometric method for the same seedlings at the same age (table II, column 4). The closeness of agreement between these two sets of data indicates that the two independent methods for assaying for cytochrome oxidase yield similar results.

Discussion

In efforts to arrive at values for the enzyme content of a tissue, the criterion of measurement must necessarily be the catalytic ability of the enzyme. For the purpose of such an assay, precautions must be taken to extract as much of the enzyme as possible, and to measure its activity under conditions shown to lead to maximal rates; for the special case of cytochrome oxidase, the activity must be extrapolated to infinite concentration of cytochrome c. Since the physiological function of cytochrome oxidase in the tissue is the catalysis of oxygen absorption, the measure of its catalytic ability in vitro can be compared to the rate of respiratory oxygen absorption. It is assumed, in a comparison of this type, that the cytochrome c in the tissue, probably by virtue of its favorable spatial distribution, does not limit the rate of respiration.

No direct attempt has been made here to answer the question regarding the relative contributions of cytochrome oxidase and other terminal oxidases to the respiration. Indeed, there is nothing in this report which serves as direct proof of the participation of cytochrome oxidase in the respiration of any of the tissues examined. Rather, we have addressed the question: Can sufficient cytochrome oxidase be extracted from a tissue to account for the measured rate of oxygen uptake of the tissue? From the results shown in table III, and to the extent to which the assay values are comparable to the actual respiratory rates, we have concluded that etiolated pea tissues, and barley and wheat shoots, at all stages of development (up to 12 days) contain amounts of cytochrome oxidase adequate to account for the respiratory rate. By contrast, the root tissues of wheat and barley do not yield the required amounts of the enzyme under the conditions of the assay. If it were shown that quantitative extraction from the tissues had been achieved, and the conditions of activity measurement were no less favorable than those under which the enzyme operates in respiration, this apparent inadequacy of cytochrome oxidase could be used as evidence that other terminal oxidases participate in oxygen absorption of these root tissues. However, it should be stressed that since even the best assay procedure is most unlikely to be 100 % effective in extracting all of the enzyme from the tissue, and transferring it without loss to the measuring vessel, the assay values reported here are to be regarded as minimal. One obvious correction which would, no doubt, lead to an upward revision of the recorded values if that for the enzyme discarded with the press cake after squeezing the mash through muslin. In this connection also, it may be mentioned that Goodwin and Waygood (8) have recently shown that the presence of hydrolytic enzymes in barley seedlings may profoundly reduce the yields of succinoxidase. This qualification must be kept in mind in those cases where the measured content of cytochrome oxidase was found to be insufficient to cope with the respiratory requirements of the tissue. And, in those cases where the assay results were in excess of respiratory needs, the conclusions are strengthened because the actual enzyme content must almost certainly be greater than that which we were able to measure.

The enzyme extracts used in this study catalyzed oxygen absorption by PPD to some degree even in the absence of added cytochrome c (figs 2 and 3). There are strong reasons for believing that the bulk of this oxygen absorption is catalyzed by cytochrome oxidase and the endogenous cytochrome c. The evidence in favor of this view is: (a) Phenoloxidase, which might be considered to be a possible catalyst for PPD oxidation, is apparently absent from these seedlings (12, 20); the feeble oxygen absorption induced by the addition of catechol to the extracts is probably ascribable to the cytochrome system, with catechol acting as a reducing agent (18). (b) For the pea extract, the activity of cytochrome oxidase at infinite cytochrome c concentration was the same whether PPD or HQ was used as the reductant. (c) The possible participation of peroxidase is unlikely, since addition of catalase did not affect the enzymatic oxidation of PPD. (d) Measurement of the cytochrome oxidase content by the manometric method provided assay values which agreed closely with those obtained by the more direct and independent spectrophotometric method; if the PPD oxidation in the absence of added cytochrome c was not mediated by the cytochrome system, consistently higher values should have been obtained by the manometric method. For example, the assay value (V_{max} per seedling) for 4-day barley seedlings is 39 by the manometric method (table II, column 4) and the corresponding value obtained spectrophotometrically is 40 (table III, column 6). Now, if the PPD oxidation observed without added cytochrome c had not in fact been due to cytochrome oxidase, then it would have been necessary to subtract this amount (which is calculated to be 16) from the manometric assay value, a correction which would have reduced the manometric value to almost one-half that of the spectrophotometric. The fact that the assay values obtained by the two methods were almost the same when such a correction was not applied, seems to justify the conclusion that endogenous cytochrome c is largely responsible for the oxygen uptake when PPD alone is added to the extract. (e) The oxida-

tion of PPD by the extracts is sensitive to cyanide and azide, which when applied at concentrations of 0.005 M to 7-day wheat root extracts, inhibited the oxygen uptake by 55 % and 50 %, respectively.

Slater (18) has already shown that endogenous cytochrome c (which may be bound, in contrast to added cytochrome c, which is soluble (3)) is much more active catalytically than added cytochrome c, due probably to its more favorable spatial relationship with respect to the oxidase. Thus, for those enzyme extracts containing relatively large amounts of endogenous cytochrome c, the subsequent addition of soluble cytochrome c to a mixture of extract and PPD would be expected to result in only small stimulations of oxygen absorption. Such as apparently the case in the barley and wheat root extracts we have examined (table I), and this consideration, no doubt, goes far toward explaining the difficulty experienced by several previous workers (1, 12, 13, 20) in attempts to detect cytochrome oxidase manometrically. Other factors contributing to the difficulty may be the failure to take precautions essential to success, such as rapid extraction of the enzyme in the cold, and provision of adequately high amounts of cytochrome c and reductant. (See also Goodwin and Waygood (8).) However, in these cases, and indeed wherever it is feasible, it seems that the spectrophotometric method of measuring cytochrome oxidase is greatly to be preferred, since not only is it more sensitive and direct, but it is independent of the endogenous cytochrome c.

Summary

Extracts prepared from etiolated seedlings of pea, wheat and barley seedlings were shown to contain cytochrome oxidase at all stages of development (up to 12 days) by both the manometric and spectrophotometric methods. When the amount of the enzyme in the tissue was evaluated in terms of activity at infinite concentration of cytochrome c, it was found that the cytochrome oxidase content of pea shoots and roots, and wheat and barley shoots, was more than adequate to account for the observed respiratory activity of the tissue from which it was derived. However, for the roots of wheat and barley, although the enzyme is present at all stages of development, the amounts extracted were not capable of catalyzing all of the respiratory oxygen absorption.

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EFFECTS OF POLLUTED ATMOSPHERES ON ORGANIC ACID COMPOSITION IN PLANT TISSUES[1]

PAUL P. MADER, GEORGE CANN AND LUCILLE PALMER
LOS ANGELES COUNTY AIR POLLUTION CONTROL DISTRICT, LOS ANGELES, CALIFORNIA

The damage to leafy vegetables caused by natural smog in the Los Angeles Basin has been estimated to amount to $500,000 yearly (8). The appearance of the field damage is characteristic and has been repeatedly described (5, 8, 9). It was also possible to produce this same damage with oxidation products of hydrocarbons plus ozone and auto exhaust plus ozone. In all experiments performed at the Earhart Laboratories of the California Institute of Technology and at the fumigation chamber of the Los Angeles County Air Pollution Control District, the damage to foliage was described from visual and microscopic observations.

The question arose whether damage to vegetation would also assert itself in a consistent *chemical change* within the plant tissues. Organic acids are known to be present in plant tissues in the form of a complex mixture. They are physiologically important because they play an important role in the plant and animal metabolism. A method described by Bulen et al (3) makes it possible to separate and to determine quantitatively a large number of individual organic acids from an acid mixture. Organic acids are also present in the Los Angeles atmosphere in significant concentrations in days of air pollution. A study of the kinds and amounts of organic acids occurring in normal healthy plants and comparison with smog injured plants suggested itself. The purpose of this investigation was to determine by chemical means whether a consistent and significant change in the organic acid composition of plants takes place as a result of their exposure to natural and synthetic pollution. Any observed change, in turn, could be expected to provide a clue to the harmful constituent(s) in the fumigating atmosphere to which the plants had been exposed.

APPARATUS AND PROCEDURES

ORGANIC ACIDS IN THE ATMOSPHERE: The presence of organic acids in the Los Angeles atmosphere was determined by passing known volumes of air through Smith-Greenberg impingers containing a 2% NaOH absorbing solution. A liquid/liquid extraction procedure over a 40-hour period was used for the analyses of the samples. A peak concentration of organic acids of 0.6 to 0.7 ppm was measured in days of intense pollution (6).

In order to determine the distribution of individual organic acids, air samples were also collected in the particulate state. Large volume samples were obtained by means of two 45 cm × 60 cm Whatman No. 43 filter papers in series through which a flow rate of 3400 l/min was maintained. The material collected on the filter papers was soxhlet extracted and the organic acids were chromatographed and individually determined by the procedure described by Bulen et al (3). The results obtained are shown in figure 1. It can be noted that formic acid was found in this atmospheric sample in fairly large concentrations. Additional samples collected in the same manner at later dates also confirmed the preponderance of formic acid among the total organic acids present in the atmosphere.

In view of the fairly high proportion of formic acid among the total organic acids, it became desirable to determine the concentrations of formic acid present in the Los Angeles atmosphere. The existing method for the determination of formic acid, as described by Grant (4) could only be employed when high concentrations were involved. Through adoption of several modifications it became possible to use the procedure for measurements of trace concentrations. The original method is based on the reduction of the formic acid with magnesium band and sulfuric acid to formaldehyde. An aliquot portion of the reduced sample is used for the formation of a unique purple complex between formaldehyde specifically and chromotropic acid. By eliminating any excess of magnesium band in the reduction step, the amount of precipitate of magnesium sulfate formed could be reduced to a minimum. The minute residue still remaining was separated from the clear supernatant liquid by centrifuging and filtering the sample. Instead of using an aliquot portion of the solution, the entire amount could be utilized, thereby considerably improving the sensitivity and accuracy of the method for low concentrations.

A number of measurements of formic acid were run on samples of the Los Angeles atmosphere. Depending on the intensity of air pollution during the sampling period, the results obtained ranged from 0.0 to 0.41 ppm by volume (table I).

The data presented in table I are typical of the results obtained from a large number of samples representing a variety of smog conditions. Most of these results pointed to a peak concentration of formic acid at approximately 0.4 ppm. This value represents from 57 to 66% of the sum total of all carboxylic acids present in the Los Angeles atmosphere on days of intense smog.

ORGANIC ACIDS IN FUMIGATED AND CONTROL PLANTS: A fumigation chamber of 11,000-liter capacity was used for the dual purpose of mixing the gases and for fumigating the test plants. The 3 walls of the chamber as well as the ceiling consisted of Plexiglas windows which are transparent in the near ultraviolet wave length region. The air rate through this chamber was 650 l/min corresponding to a 17-min turnover. The incoming air was first passed through an activated carbon filter for the removal of gaseous

[1] Received January 10, 1955.

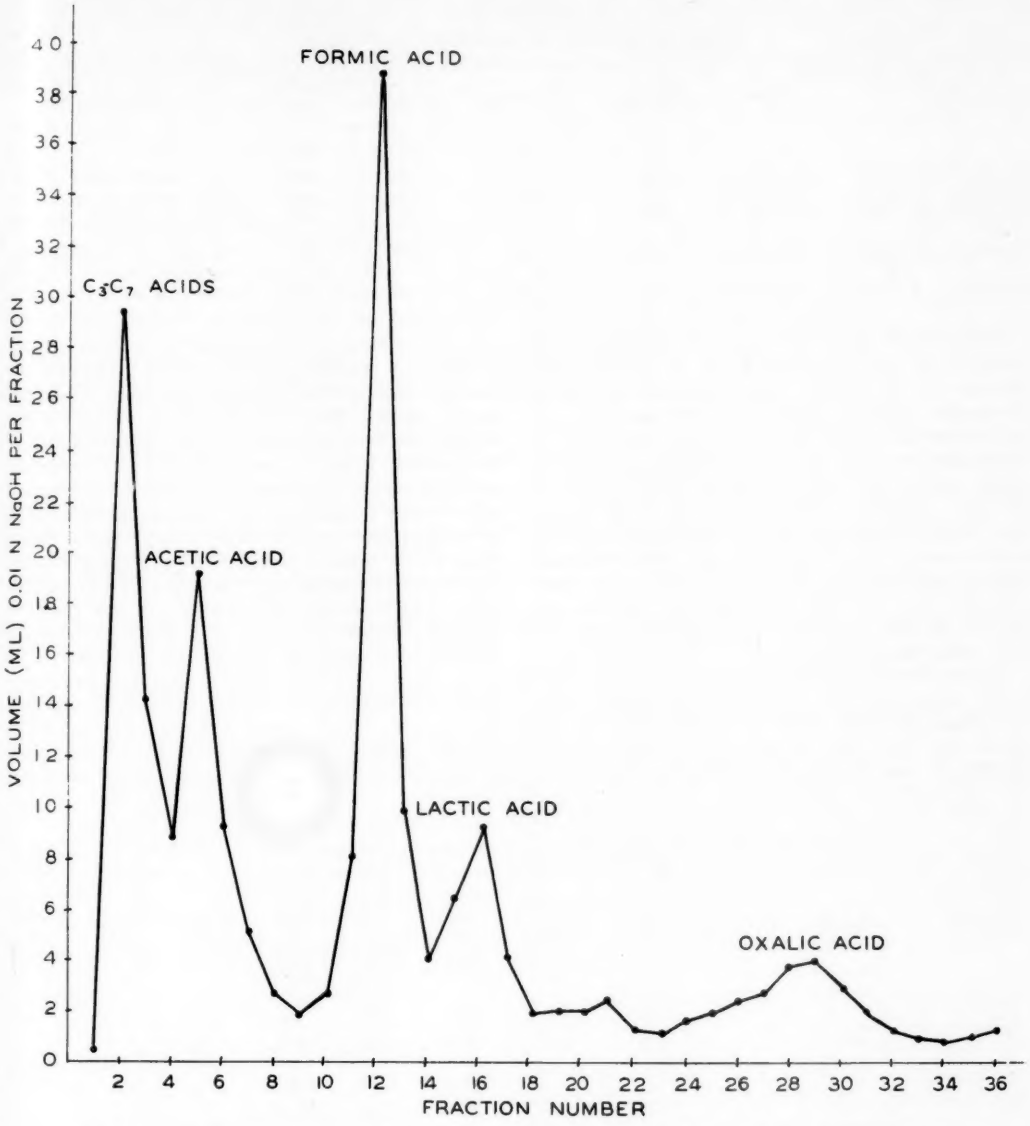

FIG. 1. Distribution of organic acids in the atmosphere collected on large blower filter papers.

contaminants from the air stream and through an evaporative type cooler to afford control of temperature and himidity.

The fumigants were introduced into the chamber through separate glass tubes with outlets 6 inches apart. A fan placed beneath the outlet directed a current of air vertically upward to insure immediate and complete dispersion of the fumigants and their reaction products. Mixing in this manner at concentrations approaching those found in the atmosphere avoided the formation of a visible aerosol such as usually results from mixing of ozone and unsaturated hydrocarbons at high concentrations. Individual hydrocarbons or gasoline vapors were introduced into the chamber by passing nitrogen gas at a constant rate through micro-impingers containing gasoline or 1-hexene. During the fumigation, the concentrations of hydrocarbons (7) and ozone (1) together with the resultant aldehydes, and formic acid, were measured in the chamber. The fumigation was carried on until a water-soaked appearance on the undersurface of the exposed plants was noticeable. This "oiliness" is usually followed by the development of a characteristic silvery or bronzen sheen.

TABLE I

FORMIC ACID LEVELS IN DOWNTOWN LOS ANGELES

DATE	TIME	R.H. AT 8:30 A.M.	VISIBILITY	SAMPLE	FORMIC ACID	REMARKS
		%	*mi*	ft^3	*ppm*	
September 22, 1952	9:15–11:15	50	⅜	12	0.37	Very intense smog
September 24, 1952	9:30–11:30	71	¼	12	0.41	Very intense smog
September 23, 1952	10:15–12:15	60	½	12	0.18	Moderate smog compared to day before
September 25, 1952	9:30–11:30	79	½	12	0.0	No smog, low visibility due to fog
February 4, 1953	9:30–11:30	94	½	12	0.15	Moderate smog

The spinach plants used in this investigation were grown in the Earhart Laboratories of the California Institute of Technology. Since amounts and ratios of organic acids are profoundly influenced by age and environmental factors, special care was taken to select only plants of the same background for fumigation and control, and to subject these plants to identical conditions after fumigation. The plants used for the fumigation and the controls were grown in the same greenhouses, under the same controlled culture conditions. All plants had received the same amount of nutrient supply during their growth, and were of the same age when the fumigation with artificial smog was performed.

Of the various methods described in the literature for the separation and identification of organic acids, the chromatographic technique by W. A. Bulen (3) offered the best potentialities. It provides for the qualitative and quantitative determination of as many as 16 biologically important acids from an acid mixture. A chromatographic column of specific dimensions was packed with silica gel from which the smaller particles had been previously removed. The column was first calibrated for one organic acid and the fraction number was determined at which the acid was eluted. Gradually the number of organic acids passed through the column was increased and the fraction at which each acid was eluted, was established. The polarity of the eluting solvent was increased by increasing the amounts of butyl alcohol in butyl alcohol–chloroform mixtures. The first developing solvent contained 5% butyl alcohol in chloroform, towards the end of the separation a 50 to 50 butyl alcohol–chloroform mixture was used. When individual fractions were titrated, a quantitative measure for the eluted acid was obtained. Before this method of separation and identification can be successfully employed, it is necessary to know be-

TABLE II

RESULTS OF CHROMATOGRAPHIC SEPARATIONS OF ORGANIC ACIDS IN PLANT TISSUES

SAMPLE NO.	TYPE OF EXPOSURE	EXPOSURE TIME	WT OF SAMPLE	FORMIC ACID DURING EXPOSURE	ACETIC ACID PER 10 GM F.W.	FORMIC ACID PER 10 GM F.W.	LACTIC ACID PER 10 GM F.W.	ACETIC ACID CHANGE	FORMIC ACID CHANGE	LACTIC ACID CHANGE
		hrs	*gm*	*ppm*	*mg*	*mg*	*mg*	%	%	%
I	1-hexene + ozone	5	4.777	0.52	8.16	3.85	4.25	+28.1	+ 75.0	−16.5
I Control	...	...	4.814	...	6.37	2.20	5.09			
II	1-hexene + ozone	5	5.952	0.67	15.52	6.99	16.42	+38.3	+ 89.5	−11.3
II Control	...	...	6.069	...	11.22	3.69	18.52			
III	Natural smog	Continuous	6.987	0.40	13.77	6.91	6.41	+ 5.0	+ 90.4	−18.9
III Control	Natural smog	Continuous	6.030	0.40	13.11	3.63	7.91			
IV	Gasoline + ozone	4	5.506	0.39	14.60	7.85	7.93	+31.8	+170.7	+15.3
IV Control	...	...	5.844	...	11.32	2.90	6.88			
V	Automobile exhaust + ozone	4	5.461	0.33	14.76	10.64	7.21	+10.9	+103.8	+17.4
V Control	...	...	5.503	...	13.31	5.22	6.15			
VI	SO_2	2	9.40	...	6.37	2.32	2.64	−17.0	0.0	0.0
VI Control	...	...	11.40	...	7.82	2.35	2.64			

forehand which acids will be encountered in a particular test sample and to calibrate the column for them. In case of spinach, a literature study indicated the organic acids identified within the plant tissues.

The preparation of the plant samples for the chromatographic analysis consisted of the following steps. The fumigated whole leaves were washed with distilled water and dried by placing them between two sheets of blotting paper. Then they were cut and placed in an Erlenmeyer flask which was immersed in a dry ice–methyl-cellosolve mixture. After a freezing time of about one hour, the fresh tissues became rigid and brittle. With the help of a glass rod flattened at the bottom the leaves were ground to a fine powder, while still immersed in the freezing solution. A known weight of the ground powder was then acidified with dilute H_2SO_4, mixed with asbestos to a homogeneous mass, placed into a glass syphon cup and extracted with purified ether for 40 hours. The acid mixture in ether solution was then made slightly alkaline, and the solvent was evaporated to dryness. The residue now present in the form of the sodium salts of the organic acids, was then reacidified with a few drops of dilute H_2SO_4 and mixed with silica gel and chloroform to a free flowing powder. The sample was now ready to be placed on top of the chromatographic column for analysis. The same procedure was simultaneously applied to the non-fumigated control plants. One sample of spinach leaves damaged by natural smog was taken in the Dominguez area on June 24, 1953. In this instance the smog damaged portions of the spinach leaves were combined to form one sample, while the undamaged portions of equal size from the same leaves formed the control. The rest of the procedure was identical with the above descriptions.

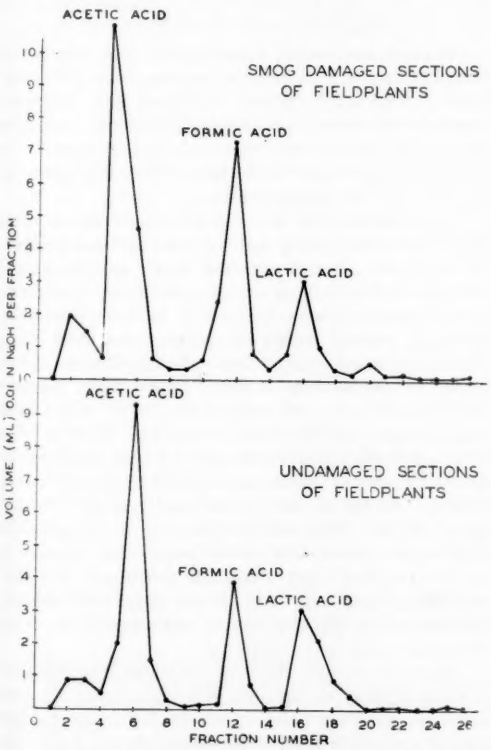

FIG. 2. Effect of natural smog on field plants.

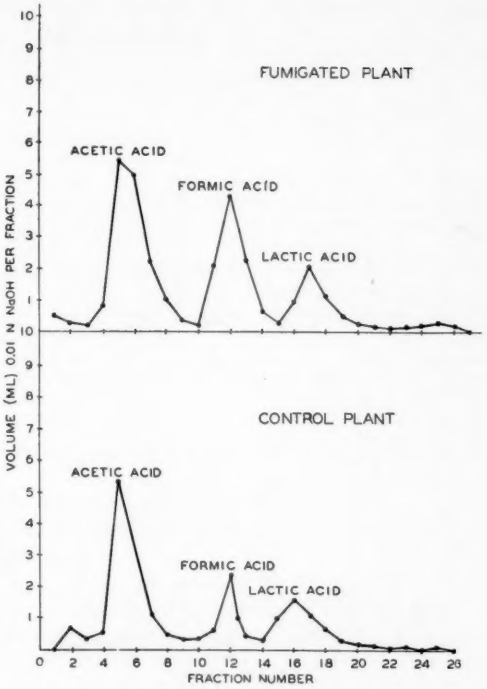

FIG. 3. Effect of gasoline vapors and ozone on plants.

DISCUSSION OF RESULTS

The results of all chromatographic analyses performed on fumigated and non-fumigated spinach leaves are compiled in table II. Comparisons between spinach plants, damaged by natural smog, gasoline vapors, automobile exhausts, and their respective controls, are shown in figures 2, 3, and 4. The abscissa of the chromatograms represents the fraction numbers, the ordinate indicates the volume of base (0.01 *N* NaOH) required to neutralize the acid contained in the particular fraction. The fractions consisted of 10 ml each, methyl-red was used as indicator.

In all chromatographic separations performed three distinct, sharp peaks are discernible between fractions 1 to 20. Previous calibrations with known organic acids indicated that acetic acid, if present, was eluted in and around fraction 5. A peak in fraction 12 and vicinity was characteristic for formic acid, while the area around fraction 16 was indicative of lactic acid. Fatty acids of the C_3 to C_7 series was eluted in and around fraction 2. They are present

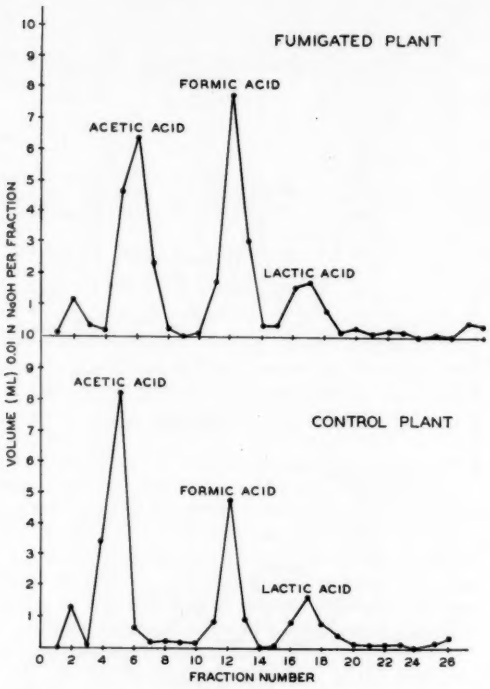

FIG. 4. Effect of automobile exhaust and ozone on plants.

only in minute amounts. In the region between fractions 20 to 78 no noticeable differences between fumigated and nonfumigated samples were found. In general, very little structure was noticed in this portion of the chromatogram when plants were used which were 4 to 5 weeks old. Only in spinach plants ready for harvesting, large quantities of oxalic acid were noticeable. Similarly, chromatogram peaks which indicated the presence of malic, citric, isocitric, and tartaric acids were noticeable in connection with mature plants.

Since absorption in fractions 11, 12, and 13 might also be due to fumaric and glutaric acids, if present, this region was also analyzed for formic acid specifically. The three fractions were combined and the chromotropic acid procedure by Grant (4) was used for the determination. This analysis was used in preference to rechromatographing the fractions in question. In all analyses performed the amount of formic acid found by chromatography was either identical or agreed very closely with the results obtained by the specific chromotropic acid method.

In two fumigations with 1-hexene and ozone, performed on plants grown at the Earhart Laboratories, an increase of 28.1 % and 38.3 % in the acetic acid content of the plants was noted. Formic acid increased by 75.0 % and 89.5 %, while lactic acid decreased by 16.5 % and 11.3 % (table II). During the fumigations the formic acid concentration in the fumigation chamber was 0.52 ppm and 0.67 ppm, respectively.

The effects of natural smog on field spinach with regard to organic acids within the plant tissues can be seen in run 3. Spinach samples were collected in the Dominguez area on June 24, 1953. The previous day an intense smog blanketed this area. The value of 0.4 ppm of formic acid in the atmosphere was not actually measured on June 23 in Dominguez, it is estimated from previously found levels on days of intense smog. Extraction as well as the chromatographic procedure used for the field plants was identical with the method used in runs 1 and 2. The comparison between the smog damaged portions of the leaves with the undamaged sections of the leaves showed an increase of 5.0 % in acetic acid; formic acid increased by 90.4 %, while lactic acid showed a decrease of 18.9 % (fig 2).

The chromatographic analysis of spinach plants damaged by the oxidation products formed from gasoline vapor and ozone showed an increase in acetic acid of 31.8 % and a 15.3 % increase for lactic acid; at the same time, the increase of formic acid amounted to 170.1 % (fig 3). The results of fumigation with automobile exhaust and ozone showed an increase of 103.8 % for formic acid, acetic acid increased by 10.9 % and lactic acid increased by 17.4 % (fig 4).

SUMMARY AND CONCLUSIONS

Organic acids were found in the Los Angeles atmosphere at concentrations ranging from 0.0 to 0.7 ppm by volume. When separated into individual acids it was noted that formic acid constituted more than 60 % of the total carboxylic acids present. Its concentration in air varied from 0.0 to 0.41 ppm, depending on the smog intensity.

Chromatographic analyses of spinach leaves, damaged by natural smog and by the oxidation products of ozone and 1-hexene, gasoline vapor, and automobile exhaust, indicated that as a result of the fumigation, a consistent and very substantial increase in the formic acid content within the plant tissues took place. These increases varied from 75 to 170 % of the amounts present in normal, comparable samples. Acetic acid increased only 5 to 38 %, while lactic acid changes varied from +17.4 to −18.9 %. The comparatively small variations of these acids do not appear to be of great significance compared to the striking change in the formic acid content of fumigated plants. This increase seems to be of particular importance because in every case when damage to plants resulted from oxidation products of hydrocarbons, formic acid was always found and actually determined in the fumigation atmospheres to which the plants had been subjected.

Based on results obtained by above investigations, a series of field fumigations is being carried out presently to determine the effects of formic acid alone, and in combination with formaldehyde, on leafy vegetables. Limits of toxicity, anatomical changes, chemi-

cal changes, and microscopic and visual description of the damage will be the subject of a second report.

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AUXIN-ANTIAUXIN INTERACTION AT HIGH AUXIN CONCENTRATIONS[1,2]

ROBERT J. FOSTER, D. HAROLD McRAE[3] AND JAMES BONNER

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, California

The growth rate of Avena coleoptile sections increases with increasing concentration of added exogenous auxin, attains a maximum, and decreases with further increase in auxin concentration. It has been shown (11, 12) that at auxin concentrations below the optimum, auxin-induced Avena coleoptile section growth is competitively inhibited by antiauxin. It has also been pointed out (12) that the inhibitory effects of antiauxin will not be completely overcome even by very high auxin concentrations because high auxin concentrations are of themselves growth inhibitory (5). It has already been found (3) that the growth inhibition caused by the antiauxin 2,4-dichloroanisole, although it is partly reversed by low auxin concentrations, is not further alleviated by high auxin concentration. Similarly, van Overbeek et al (13) have shown that the antiauxin trans-cinnamic acid, at a concentration which is nontoxic in the absence of auxin, causes toxicity symptoms in the presence of relatively high auxin concentrations. Leopold and Klein (9), who supposed that an antiauxin should antidote the inhibitory effects of high auxin concentrations have, however, reported that such growth inhibition is alleviated by maleic hydrazide, a compound which is at least qualitatively an antiauxin (9, 10, 12). The supposition that sufficiently high auxin concentrations should overcome antiauxin effects has also been made by Audus and Shipton (2).

In previous papers (5, 11, 12) the hypothesis has been advanced that the auxin molecule is bifunctional and is effective in promoting growth only after consummation of a two-point attachment to the appropriate receptor entity within the plant. It has been shown that an antiauxin is an auxin-like molecule so modified that it is monofunctional and capable only of a single-point attachment to the auxin-receptive entity. The growth inhibition induced by high auxin concentrations has further been shown (5) to possess the kinetics expected on the basis that at such high concentrations, two individual auxin molecules each combine monofunctionally with a single receptor entity. These considerations predict that growth inhibition brought about by high auxin concentrations should be enhanced by the presence of an antiauxin. Both represent mechanisms for the sequestering of auxin-receptor in the form of inactive complexes. This paper concerns the effects of antiauxin on the growth of Avena coleoptile sections in the presence of inhibitory auxin concentrations.

Materials and Methods

The materials and methods used in this investigation were similar to those used in earlier work (11, 12). Avena seedlings (var. Siegeshafer) were grown for 82 to 84 hours in vermiculite contained in stainless steel trays and maintained in a dark room under low intensity red light (with occasional orange light) at a temperature of 25 to 26° C and a relative humidity of 90 %. Only coleoptiles 2.75 to 3.25 cm in length were used. A single apical section with leaves removed and 5.0 mm in length was cut 2 to 3 mm from the tip of each coleoptile. The sections were pooled and distributed randomly in lots of 20 to individual Petri dishes. The basal nutrient medium consisted of 3 % sucrose, 0.0025 M potassium maleate buffer (pH 4.5) and Pyrex redistilled water. To this the auxins

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[3] Present address: Rohm and Haas Co., Bristol, Pennsylvania.

and antiauxins, adjusted to pH 4.5 were added to make a total volume of 20 ml. Sections were incubated in the dark at 25 to 26° C for a period of 12 hours and their length then measured under a microscope. The variability of the growth response under the present conditions has been discussed by McRae and Bonner (12).

Experimental Results

The data of figure 1 concern the effect of varying concentrations of the antiauxin, 4-chlorophenoxyisobutyric (4-CPIB), on Avena coleoptile section growth in the presence of varying concentrations of the auxin, indole-3-acetic acid (IAA). A logarithmic scale is used for the IAA concentration axis since the range in auxin concentrations involved is large, ten thousand-fold. The data of figure 1 for growth in the presence of IAA alone form a symmetrical bell-shaped curve with an optimum IAA concentration as has been earlier reported (5). The addition of increasing concentrations of 4-CPIB to the system yields a family of similar but lower curves. As is to be expected from previous data (12), 4-CPIB inhibits coleoptile section growth at suboptimal IAA concentrations and this inhibition is competitive. It is also apparent, however, that the presence of 4-CPIB (10 and 50 mg/l) causes growth inhibition at supraoptimal IAA concentrations. The growth rate in the presence of any given 4-CPIB concentrations approaches that for IAA alone as the IAA concentration is increased. It may also be noted that in the presence of the antiauxin, the optimum IAA concentration is shifted toward higher values.

Kinetics of Interaction: The data presented above, show that the antiauxin, 4-CPIB, enhances the growth inhibition caused by supraoptimal auxin concentrations.

Let us now consider the quantitative aspects of the interaction of auxin and competitive inhibitor

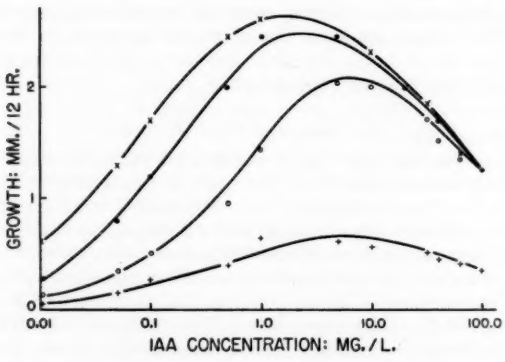

Fig. 1. Inhibition of IAA-induced Avena coleoptile section growth by 4-chlorophenoxyisobutyric acid. Each curve relates section growth rate to IAA concentration. The upper curve is that for IAA alone. The successively lower curves are for 4-chlorophenoxyisobutyric acid concentrations of 1, 10 and 50 mg/l.

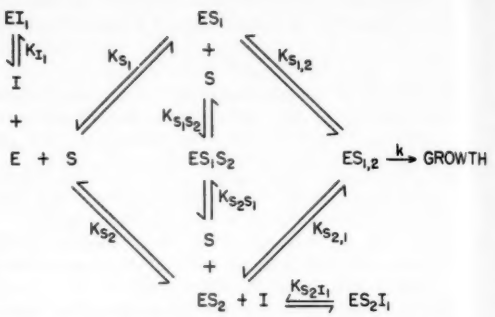

Fig. 2. Equilibria relating the formation of active receptor complex ($ES_{1,2}$) to the formation of inactive receptor-substrate complexes (ES_1, ES_2, ES_1S_2) and inactive receptor-inhibitor complexes (EI_1, ES_2I_1). The substrate, S, is bifunctional while the inhibitor, I, is monofunctional.

over a wide range of auxin concentrations. This will be done stochastically. We will first attempt to formulate the interaction in kinetic terms. The validity of the formulation may then be judged by ascertaining how closely it fits the experimental data. The system is one in which a bifunctional substrate (auxin) competes with a monofunctional (4-CPIB) inhibitor. This system may be schematically represented by an extension of that used by Foster et al (5) to describe interaction of bifunctional substrate (auxin) with receptor entity (E). The present system is depicted in figure 2. In this formulation, E may combine with S in either of two ways to form either ES_1, or ES_2, the two monofunctionally combined ES complexes. The monofunctional complexes may then rearrange internally to form the bifunctionally combined, growth-active complex $ES_{1,2}$. Alternatively, either monofunctional complex may combine with a second molecule of S to form the growth inactive complex ES_1S_2. The monofunctional inhibitor I may combine with free E to form the monofunctionally combined inactive complex EI_1. In addition, I may combine with ES_2 to form the inactive complex ES_2I_1. The dissociation constants for each of these complexes are represented by the constants K_{S1}, K_{S2}, etc. The reaction velocity, in this case growth rate, is assumed to be linearly related by the rate constant, k, to the concentration of $ES_{1,2}$.

The equation which describes substrate and inhibitor interaction for this case is of the form (Foster and Nieman, 6):

$$v = \frac{V_{ex}}{\frac{K_S}{[S]}\left(1+\frac{[I]}{K_I}\right)+1+\frac{[I]}{K_{SI}}+\frac{[S]}{K_{SS}}} \qquad (1)$$

where $V_{ex} = \dfrac{k[E]_{total}}{1+K_{S_{1,2}}+K_{S_{2,1}}}$

$$K_S = \frac{K_{S_2}K_{S_{2,1}}}{1+K_{S_{1,2}}+K_{S_{2,1}}}$$

$$K_{SS} = \frac{K_{S_2S_1}(1 + K_{S_{1,2}} + K_{S_{2,1}})}{K_{S_{2,1}}}$$

$$K_I = K_{I_1}$$

$$K_{SI} = \frac{K_{S_2I_1}(1 + K_{S_{1,2}} + K_{S_{2,1}})}{K_{S_{2,1}}}$$

It is convenient to transform equation (*1*) to a form in which v bears a linear relation to vI (equation *2*):

$$v = \frac{V_{ex}}{\frac{K_S}{[S]} + 1 + \frac{[S]}{K_{SS}}} - \frac{\left(\frac{K_S}{[S]K_I} + \frac{1}{K_{SI}}\right)}{\frac{K_S}{[S]} + 1 + \frac{[S]}{K_{SS}}} v[I] \qquad (2)$$

The slope of the line relating v to vI can be used to evaluate K_I and K_{SI} provided that K_S and K_{SS} are already known. These may be evaluated, for example, from the changes of intercepts as a function of [S]. At relatively low values of S $\left(\text{high } \frac{K_S}{[S]}\right)$ the slope term of relation (*2*) becomes:

$$-\text{slope} = \frac{1}{K_I}\left(\frac{1}{1 + \frac{[S]}{K_S}}\right) \qquad (2a)$$

At intermediate values of S (where $1 > \frac{K_S}{S}$, $1 > K_{SS}$, it becomes:

$$-\text{slope} = \frac{K_S}{[S]K_I} + \frac{1}{K_{SI}} \qquad (2b)$$

At relatively high S, $\left(\frac{S}{K_{SS}} > 1,\ \frac{K_S}{[S]K_I} < \frac{I}{K_{SI}}\right)$ the slope term of equation (*2*) becomes:

$$-\text{slope} = \frac{1}{K_{SI}}\left(\frac{1}{1 + \frac{[S]}{K_{SS}}}\right) \qquad (2c)$$

In figure 1 the data have been given in terms of a plot of v versus $\log_{10}$ (S) for several concentrations of (I). The data are re-plotted in figure 3 in terms of v versus vI for the several values of S.

Values for K_I and K_{SI} determined from the data as outlined above are given in table I. The values of

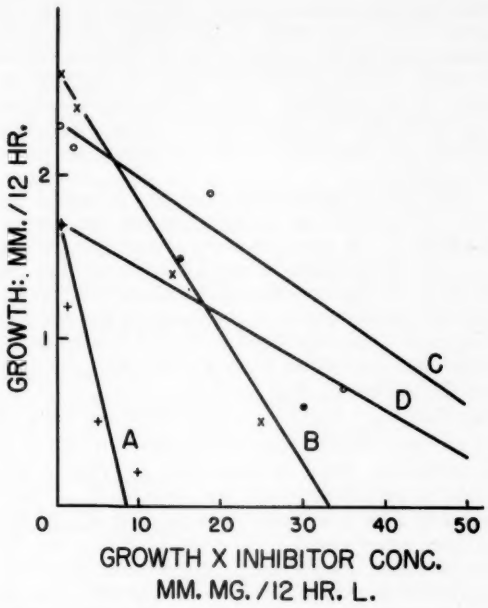

FIG. 3. Avena coleoptile section growth rate (v) as a function of growth rate × inhibitor concentration (vI). Each line represents data for a different IAA concentration. The lines are those for equation *2;* points are experimental data. A, data for S = 0.1 mg/l; B, data for S = 1 mg/l; C, data for S = 10 mg/l; D, data for S = 50 mg/l.

K_S and K_{SS} for the present data are 5×10^{-7} M and 4×10^{-4} M, respectively.

That the present data fit the formulation of figure 2 and equation *2* is shown by the fact that values for the constants K_I and K_{SI} agree closely for the three levels of S for which each may be calculated. This calculation is made from the slopes of the lines of figure 3. These lines are in turn the lines of best fit to the data as presented in figure 3. The spreads in K values are derived from the maximum and minimum slopes of lines through the experimental points.

At low concentrations of S, the amount of inhibition by I is determined principally by the K_I term. At high concentrations of S, where $\frac{S}{K_{SS}}$ becomes

TABLE I

VALUES OF DISSOCIATION CONSTANTS K_I AND K_{SI} FOR THE 4-CHLOROPHENOXYISOBUTYRIC ACID—AVENA COLEOPTILE SECTION SYSTEM AS CALCULATED FROM THE DATA OF FIGURE 3

S (IAA conc)	− Slope (− v/vI)	Equation used to calculate constants *	K_I	K_{SI}
0.1 mg/l	0.20 l/mg	*2a*	$1.2 \pm 0.1 \times 10^{-5}$ M	.....
1 mg/l	0.08 l/mg	*2b*	$1.2 \pm 0.1 \times 10^{-5}$ M	$12 \pm 1 \times 10^{-5}$ M
10 mg/l	0.034 l/mg	*2*	$1.2 \pm 0.1 \times 10^{-5}$ M	$13 \pm 3 \times 10^{-5}$ M
50 mg/l	0.028 l/mg	*2c*	.....	$12 \pm 4 \times 10^{-5}$ M

* The values for K_S and K_{SS} used in these calculations are $K_S = 5 \times 10^{-7}$ M and $K_{SS} = 4 \times 10^{-4}$ M.

large, inhibition is determined principally by the K_{SI} term. Thus, about 90% of the inhibition by I at an S concentration of 1 mg/l is due to the competitive K_I term. At an S concentration of 10 mg/l, about 80% of the inhibition is due to the uncompetitive K_{SI} term. The uncompetitive component of the inhibition by I increases rapidly as S increases above the optimum.

It has been shown earlier that the K_I values for the monofunctional inhibitory analogs of 2,4-D are of the order of 10 times or more larger than the K_S value for 2,4-D itself. It may be suspected that the K_I values for the two different monofunctional inhibitors may give estimates for the constants K_{S_1} and K_{S_2} of figure 1. Since the overall K_S for 2,4-D must necessarily equal the product $K_{S_1}K_{S_{1,2}}/(1+K_{S_{1,2}}+K_{S_{2,1}})$ and the product $K_{S_2}K_{S_{2,1}}/(1+K_{S_{1,2}}+K_{S_{2,1}})$, it follows that $K_{S_{1,2}}$ and $K_{S_{2,1}}$ would be of the order of 0.1 or less. The definition of K_{SI} above is that it is approximately equal to $K_{S_2I_1}/K_{S_{2,1}}$. Thus $K_{S_2I_1}$ would be approximately 0.1 the measured K_{SI}. As is shown in table I, the measured value of K_{SI} is just 10 times larger than K_I. The values of K_{S_2I} and K_I are, therefore, on the basis of these considerations, approximately equal. This has the physical meaning that the combination of E, the receptor entity, with I, is not greatly influenced by prior combination of E with S to form ES_2.

Discussion

It is clear that the high auxin concentration-induced growth inhibition of Avena coleoptile sections is not alleviated by added antiauxin. This inhibition, like the antiauxin inhibition of growth induced by low auxin concentrations follows quantitatively the formulations of enzyme kinetics. There have been, however, earlier suggestions that antiauxin may overcome high auxin concentration-induced effects on plant tissue. This is true of the work of Leopold and Klein (9). They first showed that maleic hydrazide behaves qualitatively as an antagonist for low (up to 1 mg IAA/l) concentrations of auxin. This property of maleic hydrazide has also been described for Avena section growth (10, 12). Leopold and Klein report that pea stem tissue becomes flaccid in the presence of high concentrations (100 mg/l) of IAA and that this flaccidity does not develop in the presence of maleic hydrazide. Pea stem sections do not, however, become flaccid within 8 hours in neutralized and buffered solutions of IAA at a concentration of 100 mg/l (7). The flaccidity observed by Leopold and Klein must, therefore, be associated with some property of their solutions other than the actual concentrations of IAA ions present. The maleic hydrazide derivative used by them apparently corrects this condition. Their results cannot be interpreted as showing that auxin-induced growth inhibition is itself overcome by maleic hydrazide.

It is known that the growth of roots is inhibited by relatively low auxin concentration (8). Inhibition of root growth is first order with respect to auxin concentration and is analogous, therefore, to the auxin-induced growth of Avena coleoptile sections rather than to the auxin-induced growth inhibition of such sections. It would be expected, therefore, that added antiauxin should antidote the auxin-induced inhibition of root growth and this has actually been found by Burström (4) and others. The fact that an antiauxin relieves auxin-induced root growth inhibition does not, however, imply that the antiauxin will or should reverse auxin-induced stem or coleoptile growth inhibition (9).

The fact that antiauxin inhibits first order auxin responses such as promotion of coleoptile section growth or inhibition of root growth, but augments second order auxin responses (such as auxin-induced growth inhibition of coleoptile sections), suggests a use for antiauxins in physiological diagnosis of auxin responses. One might determine whether a given auxin response, such as the herbicidal response, is first order or second order with respect to auxin concentration by determining whether added antiauxin suppresses or augments the appearance of the symptoms.

Summary

1. The auxin-induced growth of Avena coleoptile sections may be inhibited by high auxin concentrations or by added auxin antagonists.

2. It is shown that although the auxin-induced promotion of Avena coleoptile section growth is competitively antagonized by added antiauxin, the high auxin concentration-induced growth inhibition is not antagonized by antiauxin.

3. The interaction of antiauxin (4-chlorophenoxyisobutyric acid) and auxin (indoleacetic acid) in Avena coleoptile section growth is quantitatively that expected for interaction between a monofunctional inhibitor and a bifunctional substrate.

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THE DUAL ROLE OF AUXIN IN FLOWERING[1,2]

FRANK B. SALISBURY[3,4]

KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

The flowering of many plants is influenced by the application of auxin. Thus the photoperiodic induction of short-day plants is inhibited by applied auxin (2, 11, 13, 14, 15, 16, 17, 18, 20, 24, 25, 26, 27, 28, 30). This inhibition is a specific one in the sense that appropriately auxin treated plants not only do not flower, but continue to grow vegetatively (11, 20). Auxin in higher concentration is inhibitory to the flowering not only of short-day plants but also of many species including long-day and indeterminate forms. This inhibition is not specific to the flowering response and is accompanied by inhibition of vegetative growth (8).

The application of auxin promotes the flowering of pineapple, *Ananas*, (5, 7), of Litchi (29) and of the long-day plants, Hyoscyamus and Silene (21, 22). Auxin applied to either of these plants under threshold conditions in which the control non-auxin treated plants remain strictly vegetative, causes both the initiation of flower primordia and the characteristic elongation of the shoot axis. Leopold and Thimann (18) have found further that application of auxin to long-day plants under conditions of long day (in which the control plants flower) increases number and size of flower buds produced. There is then clear-cut evidence that the photoperiodic induction of short-day plants can be specifically suppressed by application of auxin and that the induction of long-day plants is promoted by similar auxin treatment.

In this paper, evidence will be presented which shows that flowering of Xanthium is inhibited by auxins only if applied before translocation of the flowering stimulus from the leaf is complete. Auxin applied after this time causes an increased rate of floral bud development.

METHODS

The culture of the plants was, in general, as described by Bonner and Thurlow (2). *Xanthium pensylvanicum*[5] was grown from seed of a standard strain for this purpose and maintained in a vegetative condition until time of treatment by supplementary light of ca 100 fc intensity. The day length was of 20 or more hours, total duration, including sunlight.

Experiments on the application of chemicals or of light interruption of the dark period may best be carried out with plants which have been defoliated to a single leaf. Khudairi and Hamner (12) showed and Salisbury (27) confirmed that the sensitivity of the Xanthium leaf to photoperiodic induction depends upon its size. That the half-expanded leaf is maximally sensitive and that this leaf is also the most rapidly expanding one is shown by the data of figure 1. The half-expanded leaf was defined on the basis of determinations of average leaf size for these experimental conditions, as the one between 5.9 and 9.2 cm in length, and will be referred to as the No. 3 leaf, counting toward the base from the first leaf longer than 1 cm. All of the plants in an experiment were pooled and divided into three groups on the basis of the length of the No. 3 leaf: 5.9 to 7.7 cm; 7.7 to 9.2 cm; and plants with no leaf between 5.9 and 9.2 cm (from 5 to 25 % of all plants, depending upon environmental conditions). Plants were chosen from each of these three groups to make up treatments of from 16 to 20 plants. All other leaves longer than

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[3] Atomic Energy Commission Predoctoral Fellow during part of the work reported herein.
[4] Present address: Dept. of Botany, Pomona College, Claremont, California. After July 1, 1955, Dept. of Botany and Plant Pathology, Colorado A. & M. College, Fort Collins, Colorado.
[5] Some confusion exists regarding the nomenclature of Xanthium. *Xanthium pensylvanicum* Wallr. (cocklebur) is synonymous with *X. saccharatum*, the term used by various other workers in photoperiodism. Specimens of the type of plants used in these studies have been filed at the University of California at Los Angeles Herbarium by K. C. Hamner.

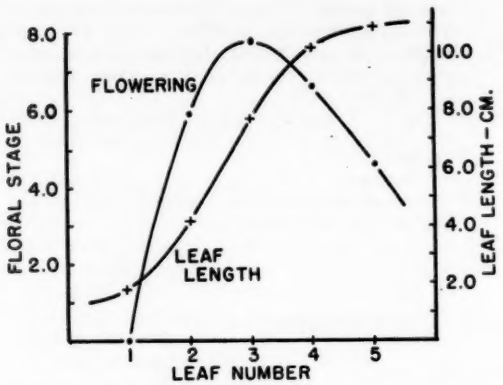

FIG. 1. The relation of leaf number (counting from the first leaf longer than 1 cm) to leaf length and to photoperiodic sensitivity. The most rapidly growing leaf (No. 3) is also most sensitive to photoperiodic induction. Plants were induced with two 12-hr nights (July 30 and 31, 1955), and dissected after 7 days.

1 cm except one were removed. In most of the experiments reported below, the remaining leaf was the No. 3 leaf, but any leaf may be used, depending upon the photoperiodic sensitivity desired for a given experiment.

The plants were induced with a single dark period, usually 16 hours in duration, unless otherwise noted.

The response of Xanthium to photoperiodic induction is strongly dependent upon light intensity (23, 27). Since light intensity varies greatly with time of year (in Pasadena, plants may be more than twice as sensitive to photoperiodic induction in June as in December), the exact date as well as special weather conditions are of importance to the interpretation of experiments with Xanthium. These data are reported below.

Temperature during induction was controlled within 2° of 23° C, although this factor is considerably less important to photoperiodic response than leaf size or time of year (23, 27). If the plants are used more than ca 32 days after planting of the seed, age is of little or no importance in photoperiodic sensitivity. A weak green light (ca 30 ergs/cm^2 × sec for 10 minutes out of every two hours during a 16-hr night), used to facilitate treatment during the dark period, was found to have a negligible effect upon flowering (27).

A System of Floral Stages

The experiments which demonstrate the promotion of floral development by applied auxin depend upon an increased rate of floral bud growth rather than upon the presence or absence of floral buds. A suitable measure of rate of bud growth has been found to be one based on a series of stages of development of the floral bud. Stage systems qualitatively similar to the present one have been used by other investigators (3, 4, 12, 15, 16, 17, 23). The stages used are illustrated in figure 2, and described in table I. Plants are dissected within two weeks after induction, and the apical bud classified. The average of the plants in a treatment will be referred to as the floral stage for that treatment.

Differences between two floral stage determinations are significant within 0.1 to ca 1.0 stages, depending upon the treatment and the number of plants within a treatment. Table II shows the results of an experiment in which five groups of 20 plants were treated with the procedure followed in the experiments reported below. In this instance, the differences in floral stage are significant within 0.2 stages. The difference required for significance increases when induction is near the threshold, or when induction is inhibited by applied auxin.

The stages are so defined that increase in stage is linear with time starting approximately 2.5 days after the beginning of induction and under optimal environmental conditions. This is illustrated in figure 3. Thus, potency of a given induction treatment may be expressed in terms of rate of floral development in stages per day.

Figure 3 shows that rate of development depends upon the degree of induction. Plants induced with inductive dark periods of varied lengths show different rates of subsequent floral development. In figure 4 the floral stage nine days after induction increases with increasing length of the inductive dark period. If conditions are not ideal, development may not be

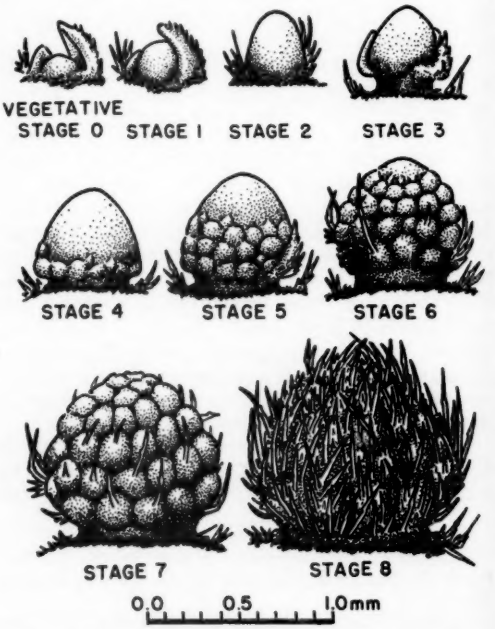

FIG. 2. Stages of development of the staminate inflorescence primordium of Xanthium (see table I). The degree of pubescence varies somewhat, depending upon experimental conditions.

exactly linear with time. The floral stage a given number of days after induction is, however, still a relative measurement of the different rates of development caused by different amounts of induction. It is used in this sense in the experiments described below.

Figures 3 and 4 show that under continuous inductive cycles, the floral buds develop at a maximum rate, regardless of the length of the inductive dark period. This maximum rate of floral development is relatively independent of environmental conditions, and hence might be taken as the standard by which the stage system is defined.

Results

Time of Auxin Application: The dual role of auxin in the flowering of Xanthium is best illustrated by experiments in which auxin is applied at various times during and following an inductive dark period. Groups of plants were treated at various times either by dipping the leaves in auxin solution or by applying 0.5 % NAA in lanolin to the stem near the tip. The time of translocation of flowering hormone out of the leaf may be measured by defoliating plants at various times after induction (12). If the flowering hormone has been translocated completely out of the leaf, the leaf may be removed and the floral bud will still develop almost as rapidly as if the leaf were left on the plant. If translocation has not occurred, removal of the leaf results in the plant remaining vegetative. Thus, the degree of flowering in groups of plants which have been defoliated at various times after induction yields a measure of the time of translocation of flowering hormone out of the leaf. In figure 5, floral stage is plotted against time of auxin application or time of defoliation. It may be seen that auxin applied during or for some time after the inductive dark period inhibits flowering. This is the inhibition reported by Thurlow and Bonner (30) and subsequent workers.

Table I

Criteria Used in Classifying Staminate Buds According to Floral Stage

Stages	Criteria
0	Vegetative. Growing point relatively flat and small, as illustrated in fig. 2.
1*	First clearly visible swelling of the growing point—hemispherical shape.
2*	Inflorescence primordium at least as high as broad, but not yet constricted at the base.
3	Inflorescence primordium constricted at the base, but no flower primordia yet visible (often some leaf primordia at the base).
4	First visible flower primordia, covering no more than the lower one-quarter of the inflorescence primordium.
5	Flower primordia covering from one to three-quarters of the inflorescence primordium.
6	Flower primordia covering all but the upper tip of the inflorescence primordium.
7	Inflorescence primordium completely covered by flower primordia. Slightly to moderately pubescent.
8	Very pubescent and usually larger than Stage 7 (diam = or > 1 mm).

*Stages 1 and 2 have been observed to revert to Stage 0 under some conditions. Hence, they are ignored or referred to as intermediates by some authors. Some induction is required, however, for the growing point to differentiate to any extent at all, and the numerals assigned to these stages have been found to be a suitable measure of this induction.

Table II

Variability in Flowering Response of Replicate Lots of Xanthium Plants

Group no.	Floral stage
1	6.15
2	6.16
3	6.10
4	6.10
5	6.20

Plants prepared according to the standard procedure. Twenty plants in each group, defoliated to a single leaf (No. 3), induced with a single dark period (16 hours—July 13, 1954), and dissected after 10 days.

Figure 5 also shows that auxin applied after the floral stimulus has been translocated out of the leaf (more than ca 45 hours after the start of induction) slightly promotes the development of the floral bud. Although the differences between controls and auxin-treated plants are small, they are significant (table II), and have been observed in 10 separate experiments. It is interesting to note that the inhibition and promotion of flowering were obtained whether the auxin was applied to the leaf or to the tip of the plant. Epinastic bending of the leaf petioles was observed in both instances, indicating that the auxin was translocated both from lamina to petiole and from bud to petiole. The fact that both inhibition and promotion of flowering by applied auxin may be demonstrated regardless of the point of auxin application, indicates that auxin concentration gradients are not responsible for the effect.

Effect of Light Intensity: The promotive effect of auxin on floral bud development may be demonstrated clearly only when light conditions during the development of the bud are suboptimal. Thus, experiments done in winter show a more pronounced promotive effect of auxin than summer experiments. That light intensity is the factor involved was shown by following the floral development of plants treated or not treated with auxin two days after induction and grown either under full greenhouse light, or greenhouse light shaded to about 25 % normal intensity with several layers of gauze. Figure 6 gives the results of such an experiment. Floral stage is plotted as a function of number of days after induction. The rate of development of non-auxin treated plants decreased with time more rapidly in the plants which were grown under low intensity light

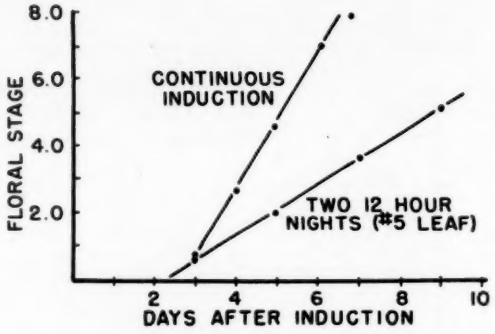

FIG. 3. The rate of floral bud development. Continuous induction was begun July 3, 1953. Two 12-hr nights were given June 6 and 7, 1953.

than in plants grown under high intensity light. A number of cloudy days resulted in slower development than normal in all plants. The buds of the auxin-treated, shaded plants, however, after a short initial lag, continued to develop at a rapid rate. Similar results were obtained when auxin was applied to the tip rather than the leaves, and also when two inductive cycles were used instead of one. These results may be interpreted on the assumption that lowered light intensity leads to a lowered auxin concentration within the plant, and that this deficit may be made up by the application of auxin. It has indeed been reported that auxin concentration in the plant is lower under short-day (6).

LOCALIZATION OF AUXIN EFFECTS: It may be shown that the inhibitory effect of auxin on induction occurs only in the leaf, but that the promotion of floral development by applied auxin occurs independently of the leaf. Groups of plants were defoliated at various times after induction. Half of the plants of each group were treated by dipping the tips in auxin solution immediately after defoliation. After an appropriate time the tips were examined, and the

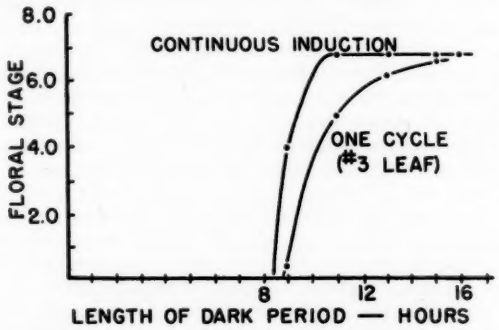

FIG. 4. Relation of rate of development of the floral bud to length of the inductive dark period. Plants were induced continuously (beginning July 9, 1953), and dissected after 6 days. Plants were induced with only one cycle (July 7, 1954) and dissected after 9 days.

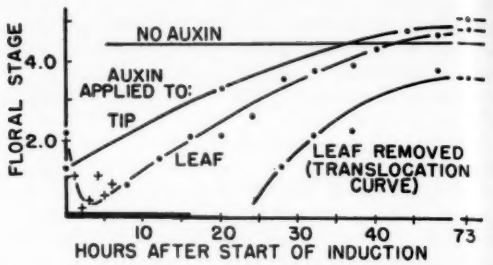

FIG. 5. The dual role of auxin in flowering. Plants were induced with a 16-hr night. Naphthalene acetic acid (0.5% NAA in lanolin) was applied to the tip. Plants were induced February 6, 1954 and dissected after 17 days. Crosses and points refer to different experiments. Points refer to auxin (10.0×10^{-4} M NAA) applied to leaves by dipping (November 6, 1953, dissected after 14 days). Crosses refer to plants treated by dipping leaves in 25.0×10^{-4} M NAA and rinsing with distilled water after 10 min (May 1, 1954, dissected after 9 days).

results are given in figure 7 (two experiments). The floral stage of the auxin-treated plants was greater than that of the controls, even though the leaf had been removed before auxin treatment. Thus, the promotive effect of applied auxin appears to be directly upon the bud. Since auxin applied before translocation of florigen from the leaf is complete, is inhibitory in the presence of the leaf (fig 5), and since auxin applied at this time gave no inhibition in the absence of the leaf, it may be concluded that the inhibitory effect of applied auxin takes place only in the leaf.

AUXIN AND ACTIVELY GROWING BUDS: It has been reported that dark periods which would be inductive for an intact plant are ineffective if all actively growing buds have first been removed (3, 4, 19). This has been interpreted by Carr as indicating that the

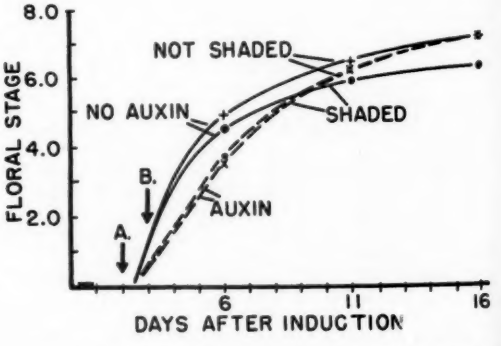

FIG. 6. The effect of auxin on floral bud development of plants grown in high or low intensity light. Plants were induced May 18, 1954 with one 16-hr night (No. 4 leaf). Plants were shaded beginning at time A. Leaves were dipped in 5.0×10^{-4} M NAA at time B.

flowering substance (referred to by him, after Gregory (9), as a hormone precursor) is not stable until it has been "fixed" in some way by the growing point. Carr suggests that if no active bud is present at the time of induction, the hormone becomes dissipated and is, therefore, not available by the time a previously inactive bud grows out. It has recently been shown that very young leaves can replace the requirement for an actively growing bud (19). It has also been shown that young Xanthium leaves are effective in the inhibition of lateral buds, from which one may infer that these young leaves produce appreciable auxin (10). These facts suggest that the absence of an actively growing bud or of very young leaves should lead to a low auxin concentration within the plant. Thus it appeared possible that the dependence of induction on an actively growing bud or on very young leaves might be a further reflection of the dependence of floral bud development on auxin. This possibility was investigated by the following experiments.

That active buds are essential to induction under the present conditions was first established by experiments in which tips were removed from groups of test plants on successive days before or after an inductive

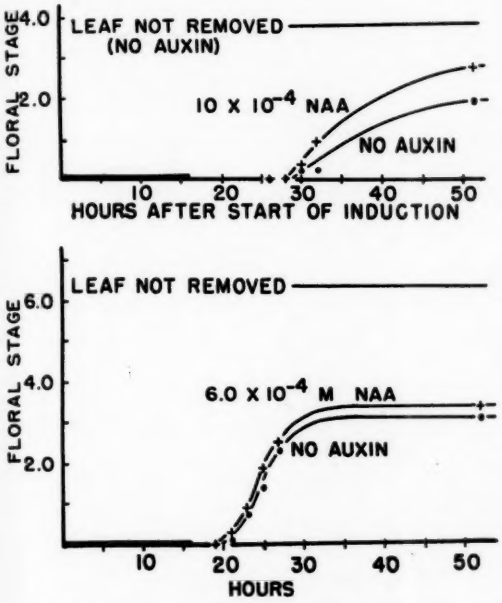

FIG. 7. Auxin promotes floral bud development when applied to the stem and tip after removal of the leaf. The two lower curves in each figure refer to plants with leaves removed. *Above.* Plants induced October 30, 1953 with one 16-hr night and dissected after 15 days; tips dipped in 10.0×10^{-4} M NAA. *Below.* Plants induced June 6, 1954 with one 15-hr night and dissected after 11 days; tips dipped in 6.0×10^{-4} M NAA and shaded during development of buds.

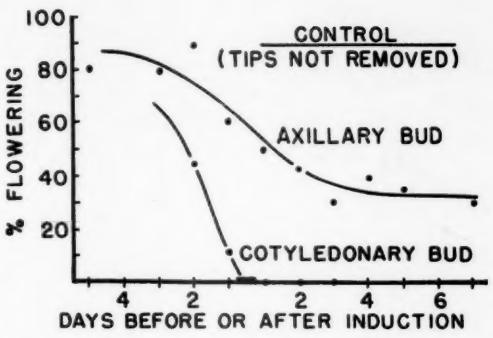

FIG. 8. Removal of the tip during or after induction decreases the flowering response. *Axillary buds:* Plants induced January 6, 1954 with one 16-hr night, and dissected after 14 days. *Cotyledonary buds:* Induced February 6, 1954 with one 16-hr night, and dissected after 17 days.

dark period. Either an axillary bud or a cotyledonary bud was left on each plant. The results are shown in figure 8, in which the percent of the remaining axillary buds which flowered is plotted against time at which the tip was removed. If the tips were cut off two or three days before induction, the axillary bud apparently became active in time to receive the flowering stimulus and differentiate into a flower bud. If the tips were removed just before or for a number of days after induction, the degree of flowering was greatly reduced. Even if the tip is removed after it has begun to differentiate (two or three days after induction—fig 3), the remaining dormant bud fails to flower when it becomes active.

To determine whether auxin can replace the requirement that active buds be present during induction, tips were removed one day before induction, and auxin in lanolin applied to the scar on successive days thereafter. All leaves and buds were removed from the plant at the beginning of the experiment except the one inductive leaf and its axillary bud. The tip, together with part of the one remaining axillary bud, was removed as shown in figure 9. This was to insure that the only meristematic tissue (later to become the growing point) on the plant was in a completely dormant condition at the time of induction. Even this method is not entirely satisfactory. In one experiment conducted in the middle of summer, the buds became active within three to four days after the tips were removed, and all plants flowered, regardless of the time the tips were removed. In another experiment, the axillary buds had begun to become active at the time the tips were removed, and insufficient meristematic tissue remained to produce new growing points in many plants. The results of two experiments conducted as outlined above are given in figure 10. In experiment A, 45 % of the plants whose tips were removed but which received no auxin, flowered. Plants which received auxin just

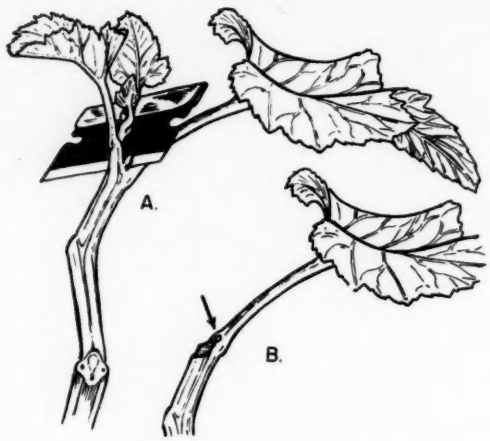

FIG. 9. Method of removing the tip so that only part of the axillary bud remains, as indicated by the arrow.

before induction were inhibited in their flowering, as is normally the case. Plants which received auxin two and three days after induction, however, flowered almost as completely as controls from which the tips had not been removed. Thus, auxin can replace the requirement for active buds in the induction of Xanthium. The data in experiment B of figure 10 show some indication that auxin is not effective if given more than about six days after induction. This may indicate that the flowering hormone is dissipated in this period of time. It was evident, however, that the meristematic tissue of some of the plants became active within six days. This may account for the flowering which did occur in many of the non-auxin treated control plants. Experiments demonstrating the replacement of the requirement for active buds with auxin have been repeated five times.

DISCUSSION

It has been shown above that applied auxin exerts two qualitatively different effects upon flowering of Xanthium. Auxin applied before, during, or for some time after the inductive dark period inhibits subsequent flowering. This inhibitive effect occurs in the leaf. Auxin applied after translocation of the flowering hormone from the leaf has been consummated promotes flowering by increasing rate of flower bud development. This effect is independent of the leaf, and appears to be exerted directly on the bud. The fact that auxin promotes floral development, as described in this paper, may aid in the interpretation of some experiments of other investigators which show a promotive effect of auxin upon flowering.

Bonner (1) and Liverman (20) were able to obtain formation of small floral buds in Xanthium by the application of antiauxins, but the buds failed to develop further unless they were subsequently supplied with auxin. Since bud development only takes place after the auxin level has been raised, this would appear to be an example of the phenomenon outlined above.

Leopold and Guernsey (15, 16, 17) have reported a promotion of flowering by low concentrations of auxin applied at low temperatures. Auxin concentrations which are too high bring about inhibition even at low temperatures. They were able to obtain this effect by soaking the seeds of long-day, short-day and day-neutral plants in auxin solutions in the cold, and also by applying low concentrations of auxin to the leaves of soybean (a short-day plant) kept on short-day at low temperature. The explanation for these results may be that low temperature prevents auxin from being effective in inhibiting the act of induction, but that there is an increase in the general level of auxin within the plant so that subsequent bud development is accelerated, as described above.

The experiments showing the requirement for active buds and its replacement by applied auxin are of interest in connection with the understanding of

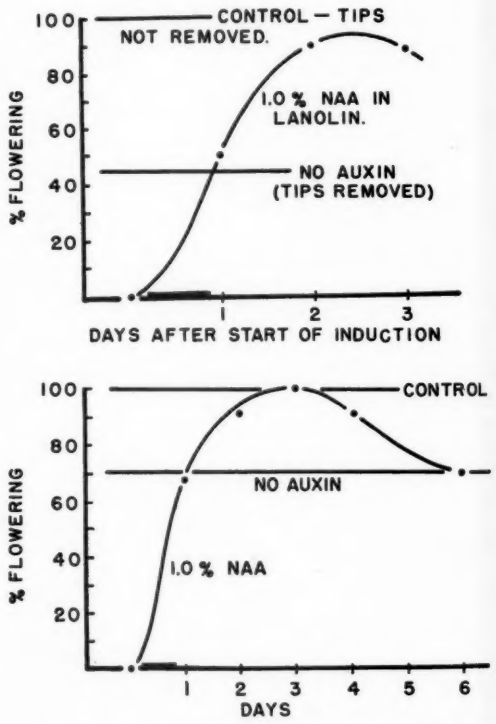

FIG. 10. Auxin applied after induction can substitute for active buds (or young leaves) which are normally required during and after induction. Tips (active buds) all removed the day before induction; auxin applied on days shown. *Above.* Induced with one 16-hr night, April 24, 1954, and dissected after 23 days. *Below.* Induced with one 16-hr night, August 21, 1954, and dissected after 14 days.

induction. Active buds are not actually required during the inductive dark period but must be present only to receive the flowering stimulus before it becomes dissipated. Thus, the requirement for active buds (or young leaves) is probably related to maintenance of induction, to the induced state, rather than to the act of induction itself.

Summary

1. In experimentation with Xanthium it is often convenient to use plants defoliated to a single leaf, although the physiological age of this leaf must be carefully controlled. The most rapidly expanding leaf is most sensitive to photoperiodic induction. Light intensity, and hence time of year, also affect the response of Xanthium to inductive treatment. Temperature, age of plant, and a weak green light used for treatments during induction are relatively unimportant.

2. A system of stages of floral bud development is described. Under optimal conditions, floral stage is proportional to time starting 2.5 days after the beginning of induction. Floral stage a given number of days after induction is proportional to length of the inductive dark period.

3. Time of auxin application experiments indicate that auxin inhibits flowering if applied before translocation of the flowering stimulus from the leaf is complete, and promotes the rate of floral bud development if applied after this time.

4. The promotive effect of applied auxin can be demonstrated most clearly when the plants are grown under low light intensity.

5. Auxin applied to the stem after defoliation, promotes bud development even though the plants may have been defoliated before translocation of the flowering stimulus from the leaf was complete. Thus, the promotive effect of applied auxin acts independently of the leaf, although the inhibitory effect occurs only in the leaf.

6. If actively growing buds are removed during or after induction, the dormant buds, when they become active, remain vegetative. Auxin applied two or three days after induction overcomes this requirement for active buds.

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ROLES OF EMBRYO AND ENDOSPERM IN DETERMINING NIACIN CONTENT OF STARCHY AND SUGARY MAIZE ENDOSPERMS[1,2]

H. J. TEAS,[3] E. G. ANDERSON and J. W. CAMERON

Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, and Department of Horticulture, University of California Citrus Experiment Station, Riverside, California

It is well established that the niacin content of maize kernels is genetically controlled. Endosperms homozygous for any one of the recessive genes sugary-1, waxy, brittle-1, brittle-2, shrunken-1, and shrunken-2 have been found to have higher niacin than normal ones (9, 10, 11 and references therein). Extensive breeding tests have shown that apart from these specific effects of single genes, the characteristic niacin levels of maize varieties may be determined by many genes acting in quantitative fashion (6). Among the specific genes associated with changes in niacin concentration, sugary-1 (su_1) has been studied the most extensively. However, the respective roles of the embryo and endosperm in determining the amount of niacin in sugary-1 and starchy-1 (Su_1) kernels have not been elucidated. The present data provide information on this problem.

Materials and Methods

The allelic genes su_1 and Su_1 will be designated in this paper by the symbols *su* and *Su*, without use of the subscript. The term "embryo" will refer to the entire embryo and adjacent scutellum. "Endosperm" refers to kernels from which the embryo has been removed, and unless otherwise indicated, includes the thin maternal pericarp which contains 2 to 3 % of the niacin of the kernel (2, 8). Embryos were removed by hand with a scalpel. Niacin assays were carried out by the method of the Association of Vitamin Chemists (1).

The maize kernels used in this study were obtained from several sources. The strain segregating for complete absence of embryo was provided by Dr. W. R. Singleton, of the Brookhaven National Laboratory. Ears from which kernels were taken to determine weights of embryos and endosperms were selected from lines of diverse genetic backgrounds maintained at the California Institute of Technology. The ears listed in table I were also obtained from such lines. The translocation B-4a ears listed in table II were produced by crossing translocation B-4a lines onto homozygous *su* seed-parent lines with the various genetic backgrounds shown. Several of these ears were supplied by Dr. Herschel Roman. Segregation of starchy and sugary kernels on such ears results from the behavior of translocations involving B chromosomes in maize (7). In the translocation employed, the distal portion of the short arm of chromosome 4, containing the *Su* gene, has been transferred to a portion of the B chromosome carrying the centromere, to form a B^4 chromosome. The remainder of chromosome 4, including the centromere, has become attached to the distal portion of the B chromosome to give a 4^B chromosome. When a plant homozygous for *su* is pollinated by one carrying translocation B-4a, about half of the resulting endosperms are sugary in phenotype. This occurs as a result of non-disjunction of the B^4 chromosome in the second microspore division, which causes one microgamete to carry two *Su* genes while the other carries one. The sugary endosperms (normally triploid) are deficient, carrying only two *su* genes (*su su* -) while the accompanying embryos are hyperploid (*Su Su su*). Starchy endosperms are hyperploid (*Su Su su su*), while the accompanying embryos are deficient (*su* -).

The mosaic-type endosperms of table III were in general of two types. Endosperms 1 through 6, in which the sugary-starchy lines of demarcation were not sharp, may have been partially "pseudostarchy" in character. Such kernels do not represent differences at the *su* locus but rather, the frequent irregular expression of modifying genes as described by

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[3] Present address: Federal Experiment Station, U. S. Department of Agriculture, Mayaguez, Puerto Rico.

TABLE I

NIACIN IN *Su* AND *su* ENDOSPERMS AND ACCOMPANYING EMBRYOS

SOURCE OF KERNELS*	NIACIN (μG/GM TISSUE)			
	EMBRYO		ENDOSPERM	
	Su	*su*	*Su*	*su*
Su KYS and *su* KYS ears ..	53.5	42.8	20.5	42.6
KYS background	22.3	18.7	17.3	40.8
Miscellaneous background**	32.0	22.0	32.4	73.6
Miscellaneous background**	39.0	29.4	26.1	36.6
Miscellaneous background**	40.3	27.7	24.9	45.0
Ear No. 1, from Teas (8) ..	18.8	23.7	17.5	35.6
Ear No. 2, from Teas (8) ..	17.5	18.4	9.5	18.9
Means	31.9	26.1	21.2	41.9

* All sources except those listed on the first line were single ears segregating both *Su* and *su*.
** Endosperm niacin levels calculated from aleurone plus inner endosperm.

Jones (3). Endosperms 7 through 11 were mosaic endosperms occurring on ears of homozygous *su* plants pollinated by *Su*. Here a sugary sector is due to the elimination of the *Su* gene from a portion of the endosperm. In these cases the sugary-starchy demarcations were sharp and clear-cut.

RESULTS

Whole kernels and endosperms homozygous for *su* usually average 20 to 30 % lighter in weight than do those carrying *Su* (5, 8). To determine whether this difference is also shown by embryos, kernel samples were taken from 12 segregating ears of varied backgrounds. *Su* and *su* kernels from each ear were dissected and the average weights of the embryos and endosperms were determined. Embryos of the 12 *su* samples averaged 39.9 mg, while those of the *Su* samples were slightly lighter, averaging 36.3 mg. The weights of the two kinds of embryos should not be considered as exact, since differences in the texture of *Su* and *su* endosperms may have caused slight systematic differences in the separation of embryo tissue. Endosperms of the 12 *su* samples averaged 145 mg in weight, whereas those of the *Su* samples averaged 214 mg. The *su* endosperms were thus 32 % lighter than the *Su* ones, which is in general agreement with the work cited above. It is apparent that the embryo weights were much less affected by the homozygous *su* gene than were the endosperm weights.

TABLE II

NIACIN IN *Su* AND *su* ENDOSPERMS FROM EARS CARRYING TRANSLOCATION B-4a

EAR NO.	GENETIC BACKGROUND OF SEED PARENT	NIACIN (μG/GM TISSUE)	
		Su	*su*
1	P 51 and CC 5 inbreds ..	32.2	60.4
2	P 51 inbred	17.9	38.9
3	*su* KYS inbred	21.5	57.6
4	P 39 and P 51 inbreds ..	26.2	31.6
5	P 39 inbred	19.9	33.0
6	Huelsen's Sweet	23.6	37.6
7	Indian Corn	15.5	42.8
8	L 317 inbred	22.8	40.3
9	P 51 inbred	23.9	62.2
10	Sweet corn	31.5	42.2
11*	Miscellaneous	27.0	57.0
12*	Miscellaneous	16.8	40.5
	Means	23.2	45.3

P = < 0.01 for the difference of the two means

* Ears 11 and 12 were segregating for separate, new occurrences of su_1, derived from irradiated seed.

TABLE III

NIACIN IN STARCHY AND SUGARY PORTIONS OF INDIVIDUAL MOSAIC ENDOSPERMS

ENDOSPERM NO.	NIACIN (μG/GM TISSUE)	
	Starchy	*Sugary*
1	14.2	18.3
2	22.1	38.0
3	21.1	23.3
4	14.3	18.7
5	9.6	13.3
6	14.7	23.6
7	24.2	35.0
8	16.4	44.6
9	14.5	35.8
10	16.7	30.7
11	14.2	37.3
Means	16.5	29.0

P = < 0.01 for the difference of the two means

Niacin levels were also determined in embryos and endosperms, separately, from paired *Su* and *su* kernel samples. Table I shows the data for 7 separate sources of kernel samples. In the first comparison, the *Su* and *su* kernels were taken from two closely related ears. In each of the others, all kernels came from a single ear. Five of the 7 *Su* embryo samples showed higher niacin levels than the corresponding *su* embryos, but the average difference was not significant as measured by the t test. For the accompanying endosperms the mean niacin value of the *su* samples was, as expected, about twice as high as for the *Su* ones, with a P value of less than 0.01.

Although the above data indicate that there is relatively little difference in the niacin levels of *Su* and *su* embryos, as compared to that found in the endosperms, it might still be possible that the genotype of the embryo controls the niacin level of the endosperm. In order to test this possibility, additional paired samples of *Su* and *su* endosperms obtained from translocation B-4a ears were employed. The *Su* endosperms came from kernels whose embryos should carry only *su* genes, while the *su* endosperms were associated with embryos which should carry *Su*. If it is the presence of a *su* embryo that conditions high niacin in the endosperm and, conversely, a *Su* embryo that conditions low niacin, then among the endosperms under study those carrying *Su* should be *high* in niacin and those carrying *su* should be *low*. Table II shows niacin data from samples of such endo-

sperms from twelve ears with diverse seed-parent backgrounds. It may be seen that the endosperm samples of *su* type averaged about twice as high in niacin as did those of *Su* type, with a P value of less than 0.01 for the difference of the two means. Thus, in *Su* and *su* endosperm, the niacin levels are determined independently of the presence of embryos of opposite genotype.

Other evidence on the relationship between embryo and endosperm was obtained from assays on individual endosperms which were composed of both starchy and sugary tissue. Table III shows the niacin concentrations determined separately on the two types of tissue from 11 endosperms. In each endosperm, niacin was higher in the sugary tissue portion than in the starchy portion. The difference of the two means of the eleven samples is significant beyond the 1 % point. It is clear that the niacin levels in this material varied primarily according to the endosperm tissue type.

The role of the embryo was further investigated by the use of certain kernels which showed reduction or complete absence of embryo. In the first case, kernels of a homozygous *su* ear segregating for a recessive " reduced embryo " mutant of radiation origin were used. Endosperms from kernels with reduced embryos averaged somewhat higher in niacin than those with normal sized embryos (37.1 μg compared to 29.0 μg/gm) even though the reduced embryos had attained only about 45 % as much weight as the normal ones. In the second case, a homozygous *Su* ear was used which showed segregation for a recessive mutant causing complete absence of embryo. Here the kernels lacking embryos also averaged somewhat higher in niacin than normal sister kernels from which the embryos had been removed; the values were 27.5 μg, compared to 22.1 μg per gm. These data demonstrate that the embryo is not essential to the production or accumulation of niacin in the endosperm.

Discussion

The evidence which has been presented indicates that the embryo in maize kernels has little to do with the niacin level of the endosperm. *Su* and *su* endosperms differ in their niacin concentrations, but this difference is correlated with their own tissue types without regard to the genotype of the embryo. Heathcote et al (2) have shown that much of the niacin of the endosperm is localized in the aleurone layer. Teas (8) demonstrated that the higher niacin in *su* endosperms is largely associated with a thicker aleurone layer than that present in *Su* endosperms. The higher niacin in the sugary areas of mosaic endosperms may be explained in the same way. Heathcote et al (2) further showed that the concentration of niacin becomes progressively lower in the inner portions of the endosperm; thus it is clear that niacin is not equalized within the endosperm by diffusion. The behavior of niacin in mosaic kernels supports this conclusion, although the pattern of niacin distribution found by the authors just cited may indicate that some diffusion occurs from the aleurone inward. Jones (4) has reported that carbohydrate differences in starchy-sugary mosaic endosperms are cell-limited as seen under the microscope, while certain genetically-controlled pigments are diffusible.

The fact that starchy-sugary mosaic endosperms have two differing levels of niacin indicates that such differences are brought about within the kernel and are not the result of differences in the vascular tissues which serve *Su* and *su* kernels. The higher niacin in sugary kernels must be due to an increased ability of sugary endosperms to take up or to synthesize more niacin, or to destroy less. The niacin relationships reported here indicate the desirability of using endosperm tissue for studies of endosperm niacin biosynthesis.

Summary

Data from a series of assays on maize kernels in which the *Su* and *su* genes occurred in various relationships indicate that the embryo plays no decisive part in determining the niacin level of the endosperm. It was shown that embryo weights from *Su* and *su* kernels were nearly equal, whereas endosperm weights averaged 32 % less in *su* than in *Su*. Niacin concentrations were not significantly different in *Su* and *su* embryos, but in accompanying endosperms the concentrations were about twice as high in *su* as in *Su*. In other kernels where *Su* endosperms were associated with *su* embryos, and vice versa, the niacin content of the endosperm was correlated with endosperm genotype and was independent of the genotype of the embryo. Among kernels whose endosperms were phenotypically mosaic for sugary and starchy tissue, the niacin level in each endosperm portion was correlated with the tissue phenotype. Kernels which carried reduced embryos and others in which the embryo was entirely absent, contained a somewhat higher concentration of niacin in the endosperm than did normal sister kernels.

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RELATION OF RESPIRATORY AND ENZYMATIC ACTIVITY TO CORN SEED VIABILITY[1,2]

GLYN O. THRONEBERRY[3] AND FREDERICK G. SMITH
DEPARTMENT OF BOTANY AND PLANT PATHOLOGY, IOWA STATE COLLEGE, AMES, IOWA

Seed germination like any other growth process must depend on respiratory metabolism. Failure to germinate, therefore, may be due to breakdown in some part of the respiratory machinery. Perhaps the strongest evidence for this relationship is the success often reported (1, 2, 7, 9, 15) in estimating viability with the tetrazolium test which is known to involve dehydrogenase systems (8, 16). Otherwise there is little information on the changes in metabolic and enzymatic activity of seeds associated with loss of viability. The most extensive enzyme work so far has been with catalase and peroxidase and the results have not established any general correlation between activity and viability (6, 11, 13).

The plan of the present work was to explore the relationship between certain indices of respiratory metabolism and viability in corn. Alcohol and malic dehydrogenases were chosen because they were found previously (16) to be especially active in tetrazolium reduction in this seed. Cytochrome oxidase was included because there was evidence that it was the principal terminal oxidase in corn (12). Manometric measurements of overall respiratory capacity also were made as a test of the general hypothesis that failure of respiratory metabolism is an important cause of loss in viability and in the search for clues to other enzymatic systems limiting viability. It was hoped in this way to provide a more systematic basis for the development of quick tests for viability. A practical quick test of this kind would depend on the breakdown of respiratory or enzymatic activity at an early stage of the germination process. The 18-hour stage in corn was chosen because the embryo cells have then become fully turgid and this is approximately the stage at which most information about the tetrazolium test is available. Freezing and drying treatments were used to produce a range of viability because these approximate adverse conditions which are encountered in production and marketing.

MATERIALS AND METHODS

Hand harvested ears were collected from three open pollinated hybrids, B6 × W22, B6 × L289, and Ia. 4297 at moisture levels of 32 to 44 %. Husked ears were frozen and/or dried to 8 to 12 % moisture under various conditions (table I) to provide lots with a wide range of germination percentages. Seeds were not disinfected because of possible danger of the fungicide affecting the metabolic properties of the excised embryos. However, lots showing surface fungal growth during germination did not have significantly higher respiratory or enzymatic activities than others of equal germination which showed no sign of contamination. Only in the case of lot 24-51a, which had visible mold damage in the embryo, was there any evidence that mold activity affected the measurements reported.

Germination tests were made with 100 seed samples on moist blotters in an alternating temperature germinator (9 hr, 30° C, 15 hr, 20° C). Vigor ratings which estimated speed as well as extent of germination were calculated as follows. The number of normal seedlings per 100 seeds counted each day was multiplied by the reciprocal of the time in days in the germinator. The values for each day were then totalled when germination was complete in 7 or 8 days. The cold test was essentially a measure of germination under adverse conditions, principally low temperature and exposure to seed-invading fungi. The method, following that of Svien and Isely (18), was as follows. Samples of 100 seeds were planted in a substrate consisting of 1/3 Pythium-infected soil and 2/3 sand, which was maintained at 60 % saturation. After 1 week at 10° C the temperature was raised to 30° C and the seedlings counted after 5 days.

Tetrazolium tests were made on 100 seed samples by soaking for 18 hours at 30° C, bisecting longitudinally, and exposing cut side down in 0.05 % 2,3,5-triphenyltetrazolium chloride in the dark for 2 hours. To be classified as viable, normal staining of the

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[3] Present address: Agronomy Department, Kansas State College, Manhattan, Kansas.

plumule, scutellar node, and central area of the scutellum was required (9).

For enzymatic and respiratory measurements seeds were soaked in water for 18 hours at 30° C, embryo side down. At this stage of germination the seeds appeared to be fully imbibed but only rarely had the radicle emerged. Embryos of 50 seeds were excised with a small spoon-shaped spatula, bisected longitudinally, and cut into approximately 0.5 mm slices which were kept in an ice bath. The slices then were washed, mixed, and blotted dry, and approximately ¼ of the sample was weighed and used for enzymatic assay, and the remainder was used for respiratory measurements. For the former, the slices were ground cold in 0.02 M, pH 8 phosphate (Na_2-HPO_4–KH_2PO_4) in a glass homogenizer to make a 10 % homogenate on a fresh weight basis. This was centrifuged cold at 1000 × g for 8 minutes to remove cellular debris, and part of the supernatant was used for dehydrogenase assay. Previous work (19) had shown that essentially all the malic and alcohol dehydrogenase activity was in this fraction. The remainder was centrifuged cold at 20,000 × g for 1 hour to sediment the particle fraction containing cytochrome oxidase. The latter was suspended in 0.02 M, pH 7 phosphate and both enzyme fractions were stored in an ice bath until assayed.

Both oxidase and dehydrogenase activity were determined by a rapid colorimetric technic, the former based on that of Ginter and Smith (4) and the latter developed earlier in the present study (19). Conditions adopted for malic dehydrogenase assay were as follows: 0.032 M, pH 8 phosphate (Na_2–K), 0.04 M, pH 8 malate, 2.9×10^{-4} M diphosphopyridine nucleotide (Schwarz), 1.08×10^{-5} M oxidized dye (2,6-dichlorobenzeneoneindo-3′-chlorophenol, Eastman), 0.1 M, pH 8 cyanide, sufficient pig heart diaphorase (19) to saturate the system, and sufficient diluted enzyme solution and water to make a total volume of 2.5 ml. The conditions for alcohol dehydrogenase were the same except for 0.14 M alcohol and 0.08 M, pH 8 semicarbazide in place of malate and cyanide. The conditions for cytochrome oxidase assay were as follows: 0.04 M, pH 7 phosphate (Na_2–K) 7.2×10^{-5} M reduced dye (4), 1.4×10^{-5} M cytochrome c, sufficient catalase to destroy H_2O_2 in the dye, and enough enzyme suspension and water to make a total volume of 2.5 ml. The stock enzyme preparations were stable for several hours at 0° C, but after dilution at 30° C they were unstable and were used within 3 to 4 minutes and 1 to 2 minutes for the dehydrogenases and oxidase, respectively.

Reaction rates were determined at 30° C by optical density readings taken at 5 second intervals for a 1 to 2 minute period (17). Four concentrations of each enzyme preparation were used and proportionality curves were plotted to determine average rates and to correct for blanks, when necessary, by extrapolation to zero enzyme concentration. For comparison with the manometric data, rates were expressed as Q values, equivalent microliters oxygen uptake per mg dry weight per hour. Dry weight of slices corresponding to the two enzyme fractions was calculated from the fresh weight of slices homogenized and the water content of slices determined on equivalent material by oven-drying at 100° C.

Respiratory activity of slices suspended in 0.05 M, pH 6 phosphate (Na_2–K) was measured in 50-ml Warburg flasks, using an O_2 gas phase in the O_2 and aerobic CO_2 measurements (Q_{O_2} and Q_{CO_2}, respectively) and purified tank N_2 for anaerobic CO_2 measurements ($Q^N_{CO_2}$). Aerobic CO_2 evolution was by the two flask direct method. Gas exchange rates were linear for the one hour observation period and no significant change in bound CO_2 was observed. All indices of respiratory capacity are expressed as Q values, based on oven-dry weight of slices after removal from flasks.

It should be recognized that this conventional technic for determining tissue respiratory capacity measures neither the respiration of the embryo in the intact corn seed nor the maximum capacity of the tissues. Removal of the embryo from the kernel and reduction to slices increased respiratory rates several fold. Furthermore, slices on wet filter paper showed further increase in O_2 consumption and CO_2 evolution of 25 % and 70 %, respectively, as compared with slices submerged in buffer, even with an O_2 atmosphere. A full understanding of the significance of these physical factors on corn embryo respiratory capacity will require further study, but for the present study of relative respiratory capacities of various seed lots the technic described proved adequate.

Results and Conclusions

The treatments and viability analyses of the 31 lots are summarized in table I. Cold test results were lower than germination percentages only in those lots dried at elevated temperatures. The same lots did not have correspondingly lower vigor ratings; so the difference in their behavior under favorable and unfavorable conditions did not appear to be due to growth rate. The variation in vigor ratings among lots germinating 98 to 100 % indicated differences in speed of germination. For all lots, except those germinating close to 0 and 100 %, of course, the tetrazolium test gave higher values than the germination test. The treatments involved in these lots apparently caused more damage to metabolic mechanisms essential to germination than to those involved in tetrazolium reduction.

The respiratory capacity and enzymatic activity data presented are the means of two analyses based on 50-seed samples. With the exception of cytochrome oxidase the 3 corn varieties used showed no significant differences in respiratory or enzymatic activity, so the data have been pooled. An estimate of variability, due largely to sampling rather than analytical technic, is given in table II, along with means for lots of high germination to indicate the levels of activity for viable seeds in the corn varieties studied.

It is first of interest to examine the changes in

TABLE I

TREATMENTS AND VIABILITY INDICES

LOT	TREATMENT	GERM %	COLD TEST % **	TETR TEST % †	VIGOR RATING *
16-51a	R. D. (room dried)	100	100	...	25.4
23-51b	R. D.	100	100	100	19.4
11-52	R. D.	100	98.5	100	27.8
7-51a	R. D.	100	97	99	26.9
12-51a	65 hrs, 40° C	100	68	100	27.3
3-51b	R. D.	99.5	100	100	24.9
7-52	18 hrs, 2° C; R. D.	99.5	99.5	100	23.2
4-51a	R. D.	99.5	98	100	21.5
15-51b	R. D.	99	99	100	20.8
8-51b	R. D.	99	96	99	22.8
12-52	68 hrs, 50° C	98.5	91	100	27.4
6-52	R. D.	98	100	99	23.1
14-51a	65 hrs, 40° C	97.5	21	99	22.3
11-51b	68 hrs, 40° C	95.5	42	100	17.8
13-51b	65 hrs, 40° C	90.5	31	100	16.2
1-52	4 hrs, −7° C; R. D.	89	88.5	95	19.7
5-52	72 hrs, 50° C	72	2.5	98	17.2
24-51a	R. D.	55	45	75	15.0
1-51a	Frozen in field; R. D.	51.5	55	62	10.7
14-52	8 hrs, −7° C; 100 hrs, 50° C	44.5	25.5	60	11.3
3-52	12 hrs, −7° C; R. D.	43	38	60	11.3
2-51b	Frozen in field; R. D.	39.5	41	54	8.6
9-52	16 hrs, −7° C; R. D.	39	44.5	67	11.5
10-52	16 hrs, −7° C; 100 hrs, 45° C	30	7.5	60	7.5
8-52	18 hrs, 2° C; 110 hrs, 50° C	29	7	50	5.2
13-52	8 hrs, −7° C; R. D.	25.5	26	33	7.8
4-52	12 hrs, −7° C; 114 hrs, 50° C	18	1.5	45	3.2
2-52	4 hrs, −7° C; 114 hrs, 50° C	9.5	0	40	1.7
5-51b	16 hrs, −7° C; R. D.	1	0	0	0.2
10-51b	16 hrs, −7° C; 74 hrs, 40° C	1	0	0	0.1
18-51b	65 hrs, 40° C	0	0	0	0

* Two 100-seed samples.
** One 100-seed sample for "51" collections, two for "52" collections.
† One 100-seed sample.

respiratory and enzymatic activity in the early stages of germination, during which the standard 18-hour sample was taken. Table III summarizes such data for a typical high germinating lot of corn. It should be pointed out that the "0 hour" sample actually represents slices from dry embryos suspended in aqueous medium for about one hour before measurements began. During this period the cells probably became fully imbibed and respiration was rapidly activated. It is assumed that "0 hour" activity was the potential respiratory capacity of the dormant seed since no further increase in respiratory rate occurred during the subsequent 2 hours in the Warburg flasks. The small increases in dry weight per embryo during the 27-hour period were of doubtful significance because of incomplete excision of scutellar tissue, especially at "0 hour" and possibly at 9 hours. Because of the lower activity of scutellar tissues, however, it could be estimated that this loss did not affect the Q values by more than 5 %, even with "0 hour" seeds. The protein nitrogen content of slices also remained essentially constant during the 27-hour period.

TABLE II

RESPIRATORY AND ENZYMATIC ACTIVITIES OF CORN EMBRYOS

	MICROLITERS PER HR PER MG DRY WT	
	A *	B *
Q_{O_2}	3.83 ± 0.42	0.27
Q_{CO_2}	2.14 ± 0.31	0.19
$Q^{N}_{CO_2}$	1.79 ± 0.20	0.15
Malic dehyd	21.1 ± 2.4	1.9
Alcohol dehyd	44.7 ± 6.3	6.5
Cytochrome oxidase	0.59 ± 0.22	0.19
RQ **	0.58 ± 0.06	
F/R **	0.86 ± 0.13	

* A. Mean ± stand. dev. for 13 lots, germ. 98 to 100 %.
B. Stand. dev. of mean diff. for duplicate samples of all lots.
** C. Mean ± stand. dev. for 27 lots, germ. above 10 %.

Respiratory capacity increased steadily throughout the 27-hour period with no marked change in RQ. In the beginning this increase was probably due primarily to activation of existing enzymes. Some cell enlargement probably occurred before 18 hours and cell division began shortly afterward (14). Though no net protein synthesis was found, it is likely that considerable enzyme formation occurred between 18 and 27 hours. With the two dehydrogenases the only significant increase in activity came in this period. Cytochrome oxidase activity, on the other hand, increased most in the first 9-hour period.

TABLE III

EFFECT OF TIME OF SOAKING ON THE RESPIRATORY AND ENZYMATIC ACTIVITY OF EMBRYO SLICES

HRS SOAKED	DRY WT PER EMBRYO * (MG)	Q_{O_2} **	Q_{CO_2}	RQ	MALIC DEHYD **	ALCOHOL DEHYD †	CYT OXIDASE †
0	23.7	1.44	0.93	0.65	15.9	35.4	0.41
9	25.5	2.30	1.34	0.58	16.2	34.2	0.71
18	26.9	3.90	2.26	0.58	16.6	38.9	0.75
27	27.9	5.32	2.85	0.54	24.7	47.4	0.81

* Dry wt are averages of 15 determinations except at "0 hour" which is 5.
** Respiratory rates are μl gas exchange per mg dry wt per hour, aver. of 3 determinations.
† Enzymatic activities are equivalent μl O_2 uptake per mg dry wt per hr, aver. of 3 determinations.

As far as the present data go, it appears that the 18-hour sample occurred at a time when activation of existing enzymes was complete and new enzyme synthesis was underway. It is also evident that substantial amounts of respiratory enzymes were already present in the dry seed.

The relationship among the metabolic and viability indices must be examined in terms of sample means of the various lots, and this is most conveniently done by the following graphical comparisons. Ultimately, it would be desirable to know the relationship between the metabolic activity of individual seeds and their ability to germinate, and some inference in this regard also may be possible. This was attempted by comparing the variation in metabolic indices among the lots with germination with what would be expected if all live seeds retained maximum metabolic activity and all dead seeds had none. This would result in a linear regression line from 0 % germination and 0 metabolic activity to 100 % germination and maximum metabolic activity, referred to hereafter as the " all or none " relationship.

Figure 1 shows that oxygen consumption of the various lots at 18 hours was closely related to the germination percentage. The major deviations from a linear correlation were with the mold damaged sample 24-51a and 3 lots, 3-52, 9-52, and 13-52, which contained a large number of seeds which initiated growth but failed to produce normal seedlings and were not counted as germinated. These seeds probably had nearly normal respiratory activity at 18 hours. With these exceptions, and on the " all or none " basis, the data indicate that individual seeds would not germinate if their respiration was much below normal at 18 hours and furthermore that nonviable seeds in most cases had little or no respiratory activity at that stage. The respiratory quotients and F/R ratios ($Q^{N}_{CO_2}/Q_{CO_2}$) varied little with germination percentage (table II) down to below 10 % where analytical errors became serious. This indicated that both aerobic and anaerobic CO_2 evolution varied with germination like O_2 consumption; so that there was no evidence of differential inhibition of CO_2 evolution and O_2 consumption mechanisms.

Figure 2 shows that malic dehydrogenase activity decreased with germination percentage in a roughly linear fashion, again with the exception of the 4 lots cited above. However, enzyme activity did not decrease to the same extent as germination percentage. This is particularly evident in the lots which had considerable enzyme activity remaining but contained few viable seeds. It appeared that individual seeds could not have lost appreciable malic dehydrogenase activity and remained viable, but at the same time nonviable seeds may have retained considerable activity.

A somewhat similar relationship between alcohol dehydrogenase activity and germination percentage is shown in figure 3, with two noteworthy differences. First, there was less evidence of enzyme activity in nonviable seeds, though some lots in the 10 to 40 % germination range had somewhat higher enzyme activity than would be expected from the germination percentage (" all or none " basis). Also, as in the case of malic dehydrogenase, the 3 lots with abnormal seeds had high alcohol dehydrogenase activity. Second, 3 lots in the region of 50 % germination had such low enzyme activity that some seeds must have suffered very severe reduction in activity without losing viability. On the whole, alcohol dehydrogenase seemed to be more sensitive than malic dehydrogenase to the conditions causing lowered viability, but there was less likelihood that it was the limiting factor.

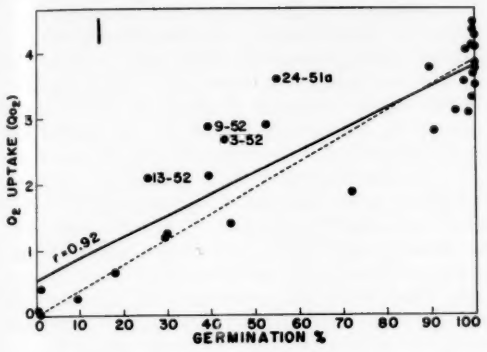
The greater variability of cytochrome oxidase in relation to germination is evident in figure 4 as well as in table II. This was partly due to varietal differences, but the data also indicated that some seeds may have had much reduced oxidase activity and still remained viable.

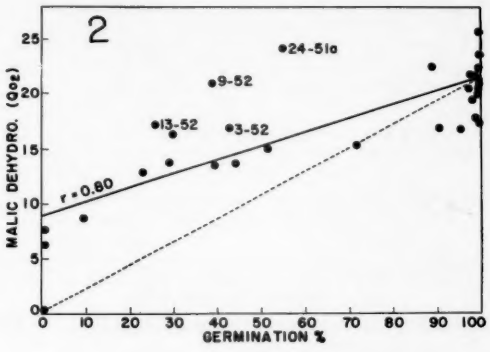
It was of interest to compare the indices of metabolic activity with the cold tests, vigor ratings, and tetrazolium tests of the various lots. No corresponding decreases in metabolic activity were found which might explain those cold test results which were lower than the germination percentages. Similarly, no evidence was found that any of the metabolic indices were more closely correlated with vigor rating (speed of germination) than with extent of germination. In the case of the tetrazolium test, however, lots which had high tests in comparison with the germination percentage also had relatively high dehydrogenase activities. This would be expected if tetrazolium reduction were limited by dehydrogenase activity.

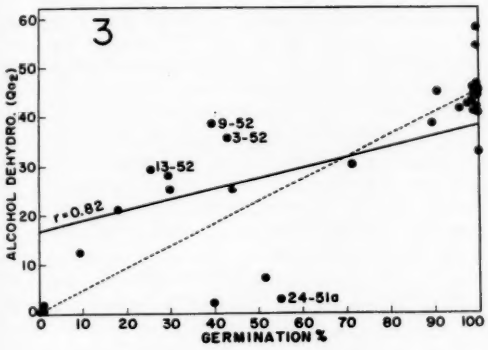
Graphical comparison of enzymatic activities and respiratory capacities also provided some evidence on the extent to which the enzymes measured may have limited respiration as well as germination. The wide variation in oxidase activity in lots of high Q_{O_2} values (fig 5) shows that cytochrome oxidase probably did not limit O_2 consumption except perhaps in lots of very low O_2 consumption. On the whole, the oxidase activity found was sufficient to support less than half the observed O_2 consumption, which indicated that it may not be the principal terminal oxidase in corn embryos.

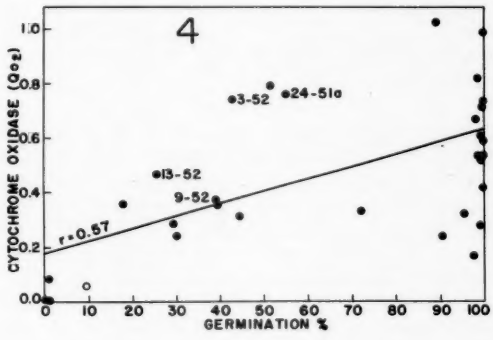
Figure 6 shows that the correlation of malic dehydrogenase activity with O_2 consumption was much higher than in the case of the oxidase, though it is clear that considerable activity must have remained in nonrespiring seeds. For this reason and because the level of malic dehydrogenase activity was many times greater than that necessary to account for the observed O_2 consumption, it seems probable that this dehydrogenase was not limiting respiration but was merely highly correlated with another enzyme or other enzymes which did.

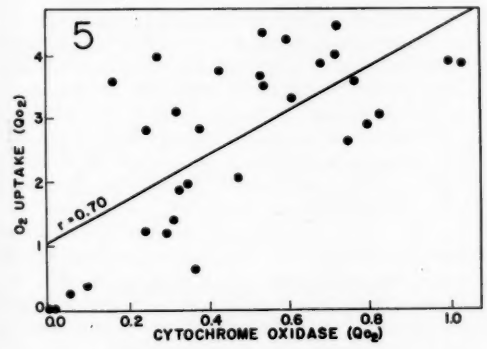
A similar comparison of alcohol dehydrogenase activity and anaerobic CO_2 evolution in figure 7 shows a rough proportionality, with the exception of the 3 lots previously mentioned which were very low in enzyme activity but in which about half the seeds were viable. As with malic dehydrogenase, the al-

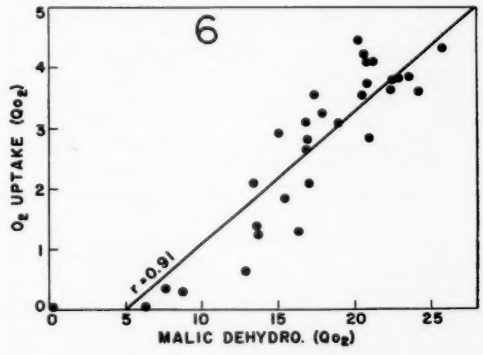












FIGS. 1–7. Each point represents mean of 2 determinations. Solid line is a regression line; broken line represents "all-or-none" relationships (see text).

FIG. 1. Relationship of O_2 consumption rate to % germination.

FIG. 2. Relationship of malic dehydrogenase activity to % germination.

FIG. 3. Relationship of alcohol dehydrogenase activity to % germination.

FIG. 4. Relationship of cytochrome oxidase activity to % germination.

FIG. 5. Relationship of cytochrome oxidase activity to O_2 consumption.

FIG. 6. Relationship of malic dehydrogenase activity to O_2 consumption.

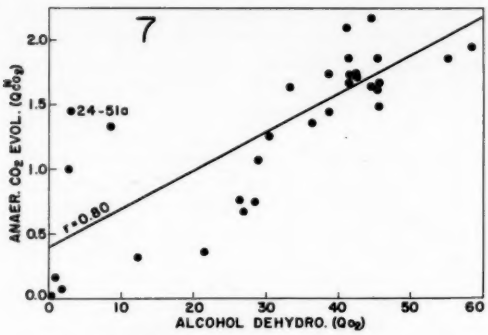


FIG. 7. Relationship of alcohol dehydrogenase activity to anaerobic CO_2 evolution.

cohol dehydrogenase activity found in almost all lots was much higher than necessary to account for anaerobic respiration, even if it were purely alcoholic. It is improbable, therefore, that this dehydrogenase was limiting.

DISCUSSION

Within the limitations of analyses using multiple seed samples, the evidence presented indicates that most seeds which failed to germinate had negligible respiratory activity at the 18-hour stage of germination. However, 3 lots contained a considerable number of seeds which apparently had nearly normal respiratory activity at this stage but did not germinate normally. The occurrence of such seeds, of course, would limit the accuracy of any quick viability test based on respiratory activity of the whole embryo at an early stage of germination.

Of the 3 respiratory enzymes measured, malic dehydrogenase activity was most closely correlated with both O_2 consumption and germination percentage. However, the high level of activity found and the pattern of variation among lots with respect to both respiration and germination made it unlikely that this enzyme limited viability in most seeds. The pattern of variation in alcohol dehydrogenase with respiration and germination was somewhat different, but again there was little evidence that this enzyme played a limiting role in metabolic processes controlling growth. The very low level of cytochrome oxidase activity and the great variability with respect to both O_2 consumption and germination indicated that the oxidase had little significance in limiting variability in the corn samples involved in this study. Not only was the oxidase activity low in comparison with O_2 consumption but it was only about 1/3 that found by Maxwell (12) by a manometric method at about the same stage of germination. The present colorimetric method had previously yielded activities equal to or greater than the manometric method on corn root tips (4) and rat organs (17) so the disagreement with Maxwell's report is probably due to varietal differences.

The relationship observed between the 2 dehydrogenase activities and germination percentages suggested a possible explanation for the higher values in the tetrazolium test than in the germination test. Dehydrogenase activity in many lots was higher than would be expected from the germination data on the "all or none" basis. In other words, dehydrogenase activity was not reduced as much, or in as many seeds, as was the activity of another enzyme (or enzymes) which did limit growth. If tetrazolium reduction, then, was controlled by dehydrogenase activity, particularly malic or alcohol dehydrogenase, the tetrazolium test would be expected to overestimate viability. Similar high tetrazolium results have been reported by Goodsel (5) and Bennett (3) with frozen corn. While such observations do not disprove the general value of the tetrazolium method, they do emphasize the caution necessary in its use where seeds may have been subjected to freezing and artificial drying.

Since cold tests measure ability of corn to germinate under adverse conditions, lots having low cold tests might be less vigorous than lots with high cold tests but with similar germination percentages. Furthermore, the former might be expected to show reduced respiratory capacity. Our tests, however, gave no evidence of relationship of cold test with vigor rating or with respiratory activity. Other factors affecting growth or susceptibility to soil fungi must have been involved. It is interesting to note that the cold test results were lower than germination percentages only in those lots which were oven dried at elevated temperatures. This is in agreement with earlier reports of Livingston (11) and others that artificial drying of seed corn caused greater susceptibility to soil organisms.

While the present results support the hypothesis that breakdown in the respiratory machinery may be an important cause of the loss of viability, it is clear that more extensive study and probably more refined technics will be necessary to determine the extent to which this is true and to locate the site of breakdown. The multiple seed samples necessary in the present technic did not allow an adequate evaluation of variation among individual seeds. It may be necessary, therefore, to employ a method for comparing enzymatic and metabolic activities with ability to grow on individual seeds. In fact, from the topographic nature of the tetrazolium test it may be desirable to confine the metabolic measurements to critical parts of the embryo. Scaling down of the present manometric and colorimetric technics appears feasible, especially with corn seeds, so that metabolic analyses might be performed on half seeds, but there is still insufficient evidence as to whether a valid growth determination could be made on the other half. Such technics, however, would be very useful in investigating the metabolic factors controlling tetrazolium reduction in embryo parts.

SUMMARY

Changes in respiratory capacity and in the activity of malic and alcohol dehydrogenase and cytochrome

oxidase of corn embryos were measured during the early stages of germination. These metabolic indices at the 18-hour stage were compared with the germination percentage, tetrazolium test, vigor rating, and cold test of 31 lots of corn of varying viability. Loss of viability appeared to be closely associated with respiratory failure in most seeds. Malic dehydrogenase activity was more closely correlated with germination percentage and respiratory capacity than that of the other 2 enzymes, although considerable malic activity was retained in nonviable seeds. It is doubtful whether inactivation of these 3 enzymes was a major cause of loss of viability, but it appeared likely that dehydrogenase activity was more closely correlated with tetrazolium reduction than with germination percentage. Variations in respiratory metabolism did not explain the differences between germination percentage and cold test or vigor rating.

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GLUCOSE DISSIMILATION IN THE HIGHER PLANT. EFFECT OF AGE OF TISSUE[1,2]

MARTIN GIBBS and HARRY BEEVERS

Department of Biology, Brookhaven National Laboratory, Upton, Long Island, New York, and Department of Biological Sciences, Purdue University, Lafayette, Indiana

We have recently described a series of experiments (4) from which it was concluded that a sequence of glucose breakdown other than that of glycolysis by the Embden-Meyerhof-Parnas (E.M.P.) pathway plays a part in the respiration of several plant tissues. There were indications from this work that an alternative method of glucose dissimilation (Warburg-Dickens, Direct Oxidation pathway) was particularly prominent in the older aerial parts of plants and by contrast the respiration of a highly meristematic tissue (corn root tip) was shown to occur exclusively by the classical glycolysis sequence (5). In this paper we will present evidence, obtained from experiments with a wide variety of plant parts of different ages that in juvenile and undifferentiated tissues generally, the E.M.P. sequence is of major importance, but that as the tissue ages, the Direct Oxidation pathway comes to play an increasingly important role.

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Materials and Methods

The method used to evaluate the relative contributions of the E.M.P. sequence and the Direct Oxidation pathway has already been described (3, 7). It involves the measurement and comparison of radiochemical yield in the CO_2 produced by equal samples of tissue respiring glucose-1-C^{14} and glucose-6-C^{14} respectively. Usually, the amounts of tissue added to the flasks were such that in a 4 to 5 hr run not more than 20 % of the glucose had been respired and we were able to calculate the C_6/C_1 ratio (% yield of C^{14} from glucose-6-C^{14}/% yield of C^{14} from glucose-1-C^{14}) in the early stages of glucose dissimilation.

Plant Materials: Root tissues of corn (Wf9 × 38-11) and Castor bean (U.S. 74) seedlings were obtained by soaking the seed overnight in water and allowing it to germinate at 29° C. When the seedlings were 6 to 7 days old and the roots were 10 to 15 cm in length they were excised and washed and cut with a razor into segments of 1 cm from the tip backwards (table I).

Stem tissue of sunflower, tomato, *Bryophyllum* sp. and Coleus was taken from stock greenhouse plants and that of pea (Alaska) was from plants grown on sand in the greenhouse. Generally three pieces of the stem were analyzed. These sections are designated in table II as A: sections from internode near the apex. B: from an internode midway between the growing tip and ground level, and C: from the internode at the base of the stem. Leaf tissue from peas, castor bean, coffee and sunflower was used. The coffee leaves were from small seedlings about one year old and the plants which were described earlier provided the rest of the leaf material. Tissues of two different ages were usually compared (table III) by selecting A: young leaves near the apex which were still small and expanding, and B: fully expanded mature leaves from older nodes. The cotyledons and hypocotyl of germinating castor beans were also examined (table IV). The stage of development of the tissue which was analyzed is recorded in the table. Another type of undifferentiated tissue used in this study was Avena coleoptile (table V). The coleoptile sections were obtained in the following manner. Husked Avena seeds ("Victory" from Svalöf) of equal size were germinated on blotters in Petri dishes. After the blotters were moistened with water, the seeds were exposed to the red light of a 60-watt bulb for 4 hours at a temperature of 24 to 26° C. After a germination period of 72 hours in darkness, 23 to 25° C and a relative humidity of 85 %, the seedling possessed coleoptiles approximately 22 mm in length. The top 3 mm of

Table I

Evaluation of C_6/C_1 Ratio for Root Tissue

Plant	Segment	Glucose-6-C^{14}	Glucose-1-C^{14}	C_6/C_1*
		% Yield	*% Yield*	
Castor bean	A (tip)	11.9	12.2	0.98 (0.98) **
7 days	B	5.7	7.4	0.77
successive	C	6.3	9.8	0.64
1 cm	D	5.6	11.2	0.50
segments	E	7.8	13.8	0.56
Castor bean				
6 days	A (tip)	8.5	9.2	0.92 (0.94)
successive	B	5.5	8.4	0.66
2 cm	C	6.9	8.9	0.78
segments				
Corn	A (tip)	20.3	16.4	1.24 (0.91, 1.12)
7 days	B	20.7	19.4	1.06
successive	C	17.7	22.9	0.77
1 cm	D	18.6	23.6	0.79
segments	E	14.7	17.7	0.83

* C_6/C_1 = % radiochemical yield from glucose-6-C^{14}/% radiochemical yields from glucose-1-C^{14}.

** The values in parentheses are from individual experiments carried out on separate occasions.

Table II

Evaluation of C_6/C_1 Ratio for Stem Tissue

Plant	Segment	Glucose-6-C^{14}	Glucose-1-C^{14}	C_6/C_1
		% Yield	*% Yield*	
Tomato	A	34.0	51.2	0.66
about 3 months	B	24.3	52.0	0.47
	C	21.4	48.8	0.44
Coleus	A	4.7	7.6	0.62
about 3 months	B	7.5	14.5	0.52
	C	4.9	9.4	0.52
Pea	A	1.2	2.5	0.48
18 days	B	1.1	2.8	0.39
	C	0.74	2.6	0.28
Pea	A	3.8	7.6	0.50
23 days	B	5.6	12.3	0.46
	C	3.3	9.3	0.35
Pea	A	5.1	11.1	0.46
23 days	C	4.2	9.9	0.42
Sunflower	A	1.1	0.95	1.15
about 2 months	C	1.4	0.99	0.71
Bryophyllum	A	2.7	3.6	0.75
about 7 months	B	6.1	8.5	0.72
	C	11.7	15.7	0.75

Table III

Evaluation of C_6/C_1 Ratio for Leaf Tissue

Plant	Segment	Glucose-6-C^{14}	Glucose-1-C^{14}	C_6/C_1
		% Yield	*% Yield*	
Castor bean	A	3.1	5.1	0.61
	B	1.4	2.9	0.48
Pea	A	3.1	7.4	0.42
21 days	B	2.0	6.3	0.32
Pea	A	5.4	9.7	0.56
24 days	AB	4.9	10.0	0.49
	B	3.4	8.7	0.39
Coffee	A	2.8	9.0	0.31
	B	1.2	4.7	0.25
Sunflower	A	6.6	9.3	0.66
about 2 months	B	2.6	4.8	0.55

the coleoptile was discarded and the next 10-mm section was used.

MANOMETRIC EXPERIMENTS: The general procedure was to cut the experimental material with a razor into sections or pieces about 1 mm in thickness. This step was omitted in the experiments with roots; these were washed in several changes of distilled water, dried lightly, and weighed into equal samples of 0.5 to 3.0 gm fresh weight. In most of these experiments four such samples of tissue were placed in the main compartments of Warburg vessels of 100 ml capacity. In addition to the tissue, the main compartment contained 30 micromoles of phosphate buffer at pH 5.0 in a volume of 2.5 to 4.0 ml of water. To two of the four vessels equal amounts (usually 10 to 30 micromoles) of glucose-1-C^{14} of known activity (7 to 30 millimicrocuries) were added while the others received the same amounts of glucose-6-C^{14}. The glucose was added immediately prior to attaching the vessels to the manometers. The vessels were shaken at 25 or 30° C for a period of 4 to 5 hours, dark conditions being provided where necessary. The atmosphere in the flask was air and the center well carried 0.2 ml 20 % KOH to absorb the respired CO_2.

MEASUREMENTS OF RADIOACTIVITY: At the end of the experiments the KOH–K_2CO_3 was removed and converted to $BaCO_3$, which was then washed and dried and assayed for radioactivity. In all the experiments C^{14} was determined by assaying $BaCO_3$.

RESULTS

In each experiment, the radiochemical yield in the respired CO_2 from each glucose sample was evaluated and this was then expressed as a percentage of the total radioactivity supplied in the original glucose. From these data, the C_6/C_1 ratio is calculated. The C_6/C_1 ratio represents the maximum fraction of glucose being metabolized by the E.M.P. pathway (7), and thus the minimum contribution of other pathways metabolizing glucose is represented by the fraction $1 - \frac{C_6}{C_1}$.

TABLE IV

EVALUATION OF C_6/C_1 RATIO OF THE COTYLEDONS AND HYPOCOTYL OF GERMINATING CASTOR BEAN

TISSUE	STAGE	GLUCOSE-6-C^{14}	GLUCOSE-1-C^{14}	C_6/C_1
		% Yield	*% Yield*	
Cotyledons	4 days	7.6	9.9	0.77
	5 days	13.6	16.8	0.81
	6 days	6.3	11.6	0.54
	7 days	1.8	3.2	0.56
	8 days	2.6	4.9	0.53
Hypocotyl	2 cm (5 days)	8.5	12.0	0.71
	5 cm	5.1	7.9	0.64
	12 cm	3.1	5.3	0.59
	13 cm	4.0	6.4	0.62
	20 cm (8 days)	4.7	8.5	0.56

TABLE V

EVALUATION OF C_6/C_1 RATIO FOR AVENA COLEOPTILE AND EFFECT OF INDOLEACETIC ACID ON THE RATIO

GLUCOSE	Mμ C ADDED	% RECOVERED	μM O_2 UPTAKE	C_6/C_1
-6-	27.7	2.77	17.0	1.13
-1-	36.9	2.45	16.0	
IAA added (2.4 mg/liter in cup)				
-1-	27.7	3.91	22.6	1.02
-6-	36.9	3.82	19.1	

Each Warburg vessel contained 18 coleoptiles of 10 mm length. After 6 hrs, control coleoptiles 12.4 mm, IAA coleoptiles 16.6 mm.

The following points are clear from the data shown in tables I to V.

a. Leaves and shoots in general show lower values for the ratio than the root tissues examined.

b. With few exceptions the ratio does not remain constant as a given plant organ ages.

c. The change in the ratio is always in the same direction, i.e., the younger tissues show higher values than their older counterparts.

d. Roots have values close to unity in the meristematic regions near the tip, but in successively older segments, the values decline rather sharply to a plateau.

e. A similar trend is obvious in the other plant parts examined but the youngest tissues which it was practicable to use frequently showed values considerably less than one.

f. The addition of indoleacetic acid to oat coleoptiles increases the consumption of glucose, but the C_6/C_1 ratio remained close to unity.

DISCUSSION

The data presented in this paper are consistent with the concept that as embryonic areas differentiate during growth, the carbohydrate metabolism undergoes a qualitative change. It appears that, while embryonic tissues respire glucose exclusively via the E.M.P. sequence,[3] the Direct Oxidation pathway makes an increasing contribution as growth and differentiation occur.

There is already evidence which, by itself, is not conclusive but which suggests that the E.M.P. sequence is particularly vigorous in meristematic tissues and that the respiratory and fermentative ability of plant cells may alter during growth. For example, Phillips (16) reported a decline in the ability of barley seedlings to accumulate alcohol under nitrogen as they grew from the 4-day to the 10-day stage, and Ruhland and Ramshorn (18) have shown that even under aerobic conditions ethyl alcohol may accumulate in young roots of *Vicia faba*, particularly in the meristematic regions. Decreases in respiratory quotient (RQ), reflecting similar changes in the contribu-

[3] We have also examined corn coleoptiles and mesocotyls which yielded ratios of 1.15 and 0.98 respectively.

tion of aerobic fermentation, may occur during plant development. Thus Merry and Goddard (15) for barley and Meeuse for peas (14) and Ruhland and Ramshorn (18) for cambia and root tips of several plants have reported RQ's considerably greater than 1 in young tisues while in the corresponding mature tissues values of near unity were the norm.

Changes in the amount of certain respiratory enzymes during the life cycle of the plant might also be considered as evidence of a changing respiratory pattern. In the pea plant alcohol and formic dehydrogenase activity decreases as germination proceeds and after six days these enzymes are virtually absent. When the pea plant reaches maturity these enzymes reappear (8).

It is clear that the relative concentrations of critical enzymes of the alternative sequences might determine the extent to which each participates in the respiration of a given tissue. Thus in the pea root the appearance of the Warburg-Dickens pathway may be related to the disappearance of aldolase, a similar situation having already been reported for the heterofermentative lactic acid bacteria *Leuconostoc mesenteroides* (9). It was the increasing evidence of the occurrence of enzymes of the Direct Oxidation pathway and the demonstration of partial reactions of this sequence in plant extracts (1, 2, 10) which prompted our investigation showing that it contributes to the respiratory breakdown of glucose (4). The interesting possibility that the non-participation of this pathway in embryonic tissues may be due to the absence of the enzyme systems is at present under investigation. However, differences in the relative amounts of the enzymes concerned may not be the universal explanation, since it sems unlikely that non-limiting amounts of the enzymes of both pathways may occur in some cells. In these cases the cause of the shunting of glucose-6-phosphate from one route to the other is unknown, although it is apparent in the case of yeast that oxygen exerts an influence on the pathway of glucose metabolism. In these cells, the E.M.P. pathway is used exclusively under anaerobic conditions (12) while in the presence of oxygen, part of the glucose is respired by the Warburg-Dickens sequence (3). A lack of oxygen may conceivably cause the same condition in "young" plant tissue. However, Ruhland and Ramshorn (18) have reported that even in the presence of 100 % oxygen, aerobic fermentation is not suppressed in some young tissue and there is no reason to believe that there is a hindrance to the entrance of oxygen into the cells of such tissue which disappears in maturation.

Until it was demonstrated that cells also contain enzymes capable of oxidizing pentose derivatives produced in the initial reactions of glucose-6-phosphate oxidation and that the Direct Oxidation pathway may, as a result, account for the complete oxidation of sugar the major significance which was attached to this sequence was a a probable route to the pentoses in vivo, the pentoses being required in the formation of ribosenucleic acid (11). It is interesting in this regard that we have been unable to detect the occurrence of the Direct Oxidation pathway in the meristematic tissues of root tips in which the utilization of pentose in such reactions might be expected to be at a maximum. Bernstein has reported a similar condition in the young chick (6). It would appear that a C_3—C_2 condensation mechanism is the major route of pentose synthesis in growing tissue. In this reaction, the C_2-component is probably transferred by transketolase from fructose-6-phosphate to glyceraldehyde-3-phosphate (C_3 piece) to yield pentose phosphate.

It has often been suggested that auxin may influence carbohydrate metabolism, and that indoleacetic acid increases the rate of oxygen uptake in coleoptiles has been verified by many investigators. In the present study, we have reconfirmed the stimulation by auxin of glucose respiration of Avena coleoptiles but found that the hormone did not affect the pathway of glucose respiration, the coleoptiles having respired the glucose in both cases exclusively via the E.M.P. pathway.

Summary

The purpose of this investigation was to study whether the developmental age of a plant tissue had any effect on the pathway of respiratory metabolism of exogenous glucose. Through the use of a method involving the measurement of the rate of glucose-1-C^{14} and glucose-6-C^{14} oxidation by the plant tissue (stem, root, leaf, cotyledon, hypocotyl, coleoptile) it was found that while immature tissue respired glucose either exclusively or to a large extent via the Embden-Meyerhof-Parnas glycolytic sequence the participation of the Direct Oxidation pathway was increasingly pronounced as the tissues aged and differentiated, and in many mature tissues it accounted for at least 50 % of the respiration. In short term experiments in which auxin stimulated the growth of coleoptile segments, there was no effect on the pathway of glucose dissimilation.

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EFFECT OF CERTAIN METABOLIC INHIBITORS ON TRANSLOCATION OF P^{32} IN BEAN PLANTS[1,2]

W. A. KENDALL[3]

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY, THE OHIO STATE UNIVERSITY, COLUMBUS, OHIO

In previous studies of the movement of solutes through phloem tissue, it has been found possible to influence the rate and direction of solute movement. In most investigations the direction of movement of certain tracers (viruses, growth substances, radioactive isotopes, etc.) was found to occur in the same direction as (by inference) the movement of organic solutes (1, 5, 8). Treatments such as subjecting the cells to temperatures of 0 to 10° and 30 to 40° C (see references in 10, 11) and also non-lethal concentrations of certain metabolic inhibitors (4), have been found to be inhibitory. More severe treatments which resulted in death of the cells were reported to have stopped the movement (11). In all of these studies it is questionable whether the observed change in solute movement resulted from an effect of the treatment on the transport mechanism per se as suggested by Curtis (4) or from an indirect effect, e.g., an alteration in the permeability of the medium through which the movement occurred, as suggested by Crafts (3). There can be little doubt, however, that these treatments influenced the metabolism of the cells through which the movement occurred, yet the role of the cytoplasm remains obscure. On the basis of all these studies, a more detailed investigation of the relationship between phloem transport and metabolism of the cells through which the movement occurs appears to be warranted. It would be of paramount importance to isolate the phases of metabolism most closely associated with the transport mechanism. From such data it might be possible to infer the role of cytoplasm relative to the movement of substances through the tissue.

The basic technique utilized in these studies was to apply a P^{32} solution to one primary leaf blade of a bean plant and subsequently measure the amount of the material which had moved through the petiole of the treated leaf and into the stem. Various chemicals which have been reported to influence certain phases of plant metabolism were injected into the treated leaf petioles.

METHODS AND MATERIALS

Bean plants (*Phaseolus vulgaris* var. Black Valentine) approximately 1.5 to 2 weeks old, were used in all of the experiments in this investigation. The plants had grown individually in one-quart Mason jars which were painted black on the outside and filled with a supplemented four-salt culture solution of the following composition: 0.0017 M KNO_3, 0.0017 M KH_2PO_4, 0.0026 M $Ca(NO_3)_2$, 0.0017 M $MgSO_4$, 2.2

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[3] Present address: Agronomy Department, University of Kentucky Agricultural Experiment Station, Lexington 29, Kentucky.

ppm H_3BO_3, 0.2 ppm $MnCl_2$, 0.9 ppm $ZnCl_2$, 0.04 ppm $CuCl_2$, 0.04 ppm MoO_3, 4.4 ppm $FeCl_3$. The culture solution was not changed or aerated during the 10- to 14-day growth period. Illumination was provided by fluorescent tubes during a 15-hr photoperiod from 5 A.M. until 8 P.M. This light source provided a light intensity of 700 fc at the height of the primary leaves. The usual temperature in the laboratory was ca 25° C.

At the time of the experimental treatments the plants were characterized by two well-expanded primary leaves, one well-developed trifoliate leaf, a second partially expanded trifoliate leaf, and a third trifoliate leaf just beginning to expand. Except for short distances at the basal and distal ends, the petioles of the primary leaves were hollow. Usually 3 to 12 parenchyma cells or several parenchyma and xylem cells separated the phloem tissue from the central cavity.

The P^{32} solution ($H_3P^{32}O_4$) was applied to the plants by the same method as that used by Swanson and Whitney (11). One 10 μl drop of a P^{32} solution was placed on the blade of one primary leaf of each plant. The drop was placed on the upper epidermis approximately 2 cm from the base of the blade and from the midvein. As a result of evaporation and absorption the drop was no longer visible on the surface of the leaf after a period of 1 to 2 hrs following the application. At that time a necrotic spot 1 to 2 mm in diameter began to appear on the surface of the leaf where the drop was placed.

The concentration of P^{32} in each of the stock solutions used was adjusted so that a 10-μl drop would contain ca 4 μc. The specific activity of the solution was approximately 0.025 mg P^{31}/mc P^{32}. Swanson and Whitney (11) reported that the absorption of P^{32} was a function of the pH when applied in a drop to one primary leaf of bean plants with the greatest absorption at low pH values. Accordingly, the pH of the stock solutions used in the investigation was adjusted to ca 1.5.

The petiole injection technique involved making a small hole into the hollow center of the petiole at the basal end, and then injecting the chemical into the opposite end until a drop of the solution was extruded from the hole at the base. A 0.5-ml tuberculin syringe with a 24 gauge needle was used to make the injections. In the preliminary experiments in which dyes were used, some of the dye could be detected in the leaf blade shortly after the injection was made in the petiole. Therefore, to minimize any influence the chemical might have on absorption and distribution of the P^{32} within the leaf blade, the petiole injections were made 1 hr after applying the P^{32} solution. Earlier experiments by Swanson and Whitney (11) indicated that relatively little P^{32} moved out of the leaf within the first hour after application.

To avoid any possible effects of these inhibitors on the process of photosynthesis, and, thus, indirect effects on either absorption or translocation, the lights were turned off immediately after the injections were made, and the plants kept in the dark for the remainder of the treatment period. It was also possible for some of the injected chemical to move into the stem, roots, or other leaves of the plant and influence metabolism in regions other than in the treated leaf. Such changes in metabolism at sites remote from the P^{32} treated leaf might result in differences in the rate of translocation through the phloem cells of the treated leaf petiole, possibly by changing the phosphorus gradient to the areas of accumulation. In order to distinguish between this indirect influence of the chemical on translocation of P^{32} and the direct effect of the chemical when in contact with the phloem cells, some injections were made into the petiole of the primary leaves opposite to the ones previously treated with the P^{32} solution. Any differences, then, between the results of these two treatments should be due to the direct effect of the chemical on the movement of P^{32} when in contact with the cells through which the movement occurs.

Four hours after the application of the P^{32} solution, the plants were harvested. The roots, hypocotyl, stem, and trifoliate leaves were harvested in one fraction and the P^{32} treated leaf, including the petiole, was harvested in a second fraction. In preliminary experiments no P^{32} was detected in the primary leaf opposite to the P^{32} treated leaf, and therefore, the former was not included in subsequent assays. Treatment comparisons were based upon the assay values of the root, stem, hypocotyl, and trifoliate leaves fraction which served as an index to the amount of P^{32} translocated through the petiole of the P^{32} treated leaf.

The amount of absorption of P^{32} by the treated leaves was an inherent variable and was also influenced by some of the treatments. In evaluating the latter variable, it was necessary to estimate the total amount of P^{32} which was absorbed. This was accomplished by cutting a disk 1 cm in diameter from the blade of the leaf, taking care that the necrotic spot was at the center of the disk. This excised disk and the primary leaf opposite to the P^{32} treated leaf were discarded at the time the plants were harvested. The counts per minute obtained by assaying the P^{32} treated leaf (minus the actual treated area) plus the counts per minute obtained by assaying the roots, hypocotyl, stem and trifoliate leaves fraction may then serve to index the total amount of P^{32} absorbed into the blade. It does not include, however, the amount absorbed into the leaf but not moved outside the 1 cm area.

The harvested plant material was prepared for assaying for radioactivity by drying in an 80° C oven for 12 hrs followed by partial digestion in hot, concentrated HNO_3, and drying the digested material. The plant material was placed in 50-ml beakers at the time of harvest, and in most experiments was prepared for assaying and counted in the same beaker. Counts obtained from samples in beakers were closely correlated ($r = 0.992$) with counts of the same material in 1-inch stainless steel cupped planchets. A con-

ventional end-window counter and scaler circuit was used for counting.

In some preliminary experiments the quantity of P^{32} applied to the primary leaves was varied to study the correlation between the amount of P^{32} absorbed by the primary leaf and the amount subsequently translocated to the stem. A highly significant positive correlation was obtained between the total amount of P^{32} absorbed by the plants and the amount translocated through the petiole of the P^{32} treated leaf. Therefore, the translocation data in the following tables are presented as percentages of the total P^{32} which was absorbed.

Results and Discussion

Metabolic Inhibitors, *2,4-Dinitrophenol:* 2,4-Dinitrophenol (DNP) was used in three experiments at concentrations of 0.002 and 0.005 M at a pH of 3.5. The check solution consisted of distilled water adjusted to a pH of 3.5 with HCl. A summary of the results of these experiments is given in table I. The differences in the average total amounts of the tracer which was absorbed by the plants were not consistently correlated with any one treatment throughout the 3 experiments nor significantly different at the 0.05 level of significance.

Differences between means of the percentages of the total absorbed tracer translocated to the stem were consistently observed. A reduction in the average percentage value of approximately 75 % occurred in each of the 3 experiments when DNP at a concentration of 0.005 M was injected into the petiole of the P^{32} treated leaf. No reduction occurred when this chemical at the same concentration was injected into the petioles of primary leaves opposite to the P^{32} treated leaf. Thus, the transport of P^{32} was retarded only when DNP was in contact with the phloem cells through which the migration was occurring.

Table I

Total P^{32} Absorbed and Percentage of the Total Translocated to the Stem of Plants in Which 2,4-D Was Injected into the P^{32}-treated Leaf or Its Opposite Leaf*

Expt no.	Conc	Total P^{32} absorbed		P^{32} translocated to stem	
		Treated leaf	Opposite leaf	Treated leaf	Opposite leaf
	M	*cpm*	*cpm*	%	%
1	0	2,400	..... **	57	..
	.002	900	.....	22	..
	.005	2,300	1,600	11	49
		L.S.D. 5 %—1,200		L.S.D. 5 %—17	
2	0	62,000	.....	24	..
	.002	42,000	.....	12	..
	.005	48,000 †	71,000 ††	6 †	29 ††
		L.S.D. 5 %—48,000		L.S.D. 5 %—13	
3	0	83,000	.....	14	..
	.002	100,000	94,000	13	12
	.005	85,000	100,000	4	15
		L.S.D. 5 %—51,000		L.S.D. 5 %—10	

* Eight plants per treatment.
** No treatment.
† Six plants per treatment.
†† Seven plants per treatment.

Table II

Total P^{32} Absorbed and Percentage of the Total Translocated to the Stem of Plants in Which NAF Was Injected into the P^{32}-treated Leaf or Its Opposite Leaf*

Expt no.	Conc	Total P^{32} absorbed		P^{32} translocated to stem	
		Treated leaf	Opposite leaf	Treated leaf	Opposite leaf
	M	*cpm*	*cpm*	%	%
4	0	38,000	..... **	28	..
	.12	38,000 †	.....	12 †	..
	.25	30,000 ††	38,000 ‡	7 ††	28 ‡
		L.S.D. 5 %—20,000		L.S.D. 5 %—14	
5	0	13,000	.....	27	..
	.18	28,000	.....	10	..
	.35	36,000	26,000	11	22
		L.S.D. 5 %—22,000		L.S.D. 5 %—10	
6	0	25,000	.....	32	..
	.10	46,000	.....	32	..
	.20	32,000	50,000	25	47
		L.S.D. 5 %—23,000		L.S.D. 5 %—14	

* Eight plants were used for each treatment in expts 4 and 5, and 7 plants per treatment in expt 6.
** No treatment.
† Six plants per treatment.
†† Seven plants per treatment.
‡ Five plants per treatment.

Sodium Fluoride: Sodium fluoride was injected into the petioles at various concentrations from 0.10 to 0.35 M (see table II). The pH of the solutions used in each experiment was adjusted to 4.5 with HCl.

Results of these experiments are given in table II. The effects of the sodium fluoride treatments in experiments 4 and 5 on the translocation of P^{32} were very similar to those reported for DNP. Considerable inhibition resulted when the chemical was placed in the vicinity of the phloem cells through which the transport took place and very little inhibition when placed outside this region, i.e., in the opposite petiole. In experiment 6 the observed inhibition, as compared with the water control, was of lesser magnitude and was not statistically significant. However, in this experiment the transport of P^{32} was significantly retarded as compared with the results obtained when fluoride was injected into the petiole of the leaf opposite to the P^{32} treated leaf. Thus, fluoride, when in contact with the cells through which transport occurs, inhibited the movement of P^{32}.

Sodium Arsenite: Sodium arsenite was used in two experiments at concentrations ranging from 0.001 to 0.1 M at pH 4.7. The high order of variability en-

countered in these two experiments makes any conclusion questionable. Translocation of the tracer through the petiole of the P^{32} leaf was apparently inhibited in the first experiment but not in the second.

Sodium Fluoroacetate: Sodium fluoroacetate was used in two experiments at concentrations of 0.03 and 0.15 M at pH 5. A significant increase in total absorption values was obtained in one experiment, but these were not duplicated in the second experiment. There was no difference in the translocation values between the controls and treated plants in either experiment.

Growth Regulators: Because of the widespread natural occurrence of growth regulators and the obvious importance of these substances on various plant processes, several of these chemicals were used in the present investigation. The chemicals used were 3-indoleacetic acid and 2,4-dichlorophenoxyacetic acid, each at concentrations of 10, 50, and 100 ppm, and 2,3,5-triiodobenzoic acid at concentrations of 50, 100, and 150 ppm. Each chemical was used in 3 experiments. No consistent relationship was found between the concentration of injected chemical and the total absorption values, nor were any consistent trends obtained in the translocation measurements.

P^{32} TRANSPORT: The transport of P^{32} was inhibited when either DNP or sodium fluoride was placed in contact with cells through which the movement occurred. There is some evidence (2, 7, 9) that permeability of certain plant cells may be increased by these chemicals. It remains questionable as to whether the results reported by the latter authors would apply to phloem cells and whether such conditions would be conducive to or inhibit the transport of phosphorus. As metabolic inhibitors DNP and sodium fluoride are known to influence the formation and/or utilization of adenosinetriphosphate (ATP) (6). However, in view of the negative results obtained with sodium fluoroacetate and the non-conclusive results with sodium arsenite, any relationship between ATP and P^{32} transport is uncertain.

SUMMARY

Studies were initiated in an effort to isolate the phases of metabolism most closely associated with the transport mechanism. The basic technique utilized was to apply a P^{32} solution to one primary leaf blade of a bean plant and subsequently measure the amount of the material which had moved through the petiole of the treated leaf and into the stem. Various chemicals which might be expected to influence the metabolism of plant cells were injected into the treated leaf petioles.

The translocation of P^{32} was inhibited when dinitrophenol or sodium fluoride was placed in the vicinity of the phloem cells through which the transport occurred. There appeared to be little or no effect on translocation of P^{32} from treatments with sodium fluoroacetate, 2,4-dichlorophenoxyacetic acid, indoleacetic acid, or triiodobenzoic acid. The results of treatments with sodium arsenite were not conclusive.

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INCORPORATION OF RADIOACTIVE AMINO ACIDS INTO THE PROTEINS OF PLANT TISSUE HOMOGENATES[1,2]

GEORGE C. WEBSTER

KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

It has been established (2, 12) that intact tissues of higher plants readily incorporate C^{14}-labeled amino acids into their cellular proteins. In addition, evidence has been presented (11) that an isolated mitochondrial fraction from bean seedlings is capable of incorporating several amino acids. This incorporation into mitochondrial protein is of particular interest as it closely resembles the synthesis of simple peptides (14, 15, 16) in its dependence on the respiratory energy of adenosinetriphosphate (ATP). The ability of such an isolated cellular fraction to incorporate amino acids makes a further study of cell-free systems of interest.

The sole purpose of the present investigation has been to study in some detail the incorporation of amino acids into the proteins of cell-free extracts of higher plants. The present communication reports results on the nature of the incorporation process and on the partial purification and properties of a particulate system from pea seedlings that incorporates amino acids at relatively high rates.

EXPERIMENTAL

Seedlings were grown under weak red light at 26° C and 90 % humidity as described previously (13). Tissue homogenates, prepared in either sucrose-phosphate (13) or other buffers (10), were also made as described earlier. Sedimentation of cellular fractions was carried out with the techniques previously used in the study of glutamine synthesis (10). Experiments were performed in a Dubnoff Metabolic Incubator at 38° C and a shaking rate of approximately 100 cycles per minute. The basic reaction system consisted of the homogenate or cellular fraction plus 0.05 M phosphate buffer (pH 7.5) and 0.01 M glutamate-2-C^{14} or other C^{14}-labeled amino acid. The supplemental amino acid mixture used in certain experiments was composed of 0.1 mg per ml of each of the following L-amino acids: alanine, arginine, aspartate, cysteine, glutamate, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, and valine, plus 0.001 mg per ml of tyrosine. After incubation, proteins were precipitated and washed as described before (12). Radioactivity of each fraction was determined as previously (12) with a thin mica window Geiger-Müller tube and scaling circuit. Each value in the accompanying tables represents the average of eight separate determinations. The variation between samples was less than 5 % of the average value in every case. Approximately 0.1 to 1.0 % of the added glutamic acid-C^{14} or other labeled amino acid was taken up by the protein (depending on the conditions listed in the accompanying tables).

RESULTS

As is evident from table I, disruption of the cells of pea seedlings does not stop the incorporation of radioactive glutamate into the total protein of these cells. However, the medium used in the preparation of the homogenate exerts a marked effect on the rate of incorporation. In agreement with previous studies on organized oxidative and phosphorylative activities in plant homogenates (1, 6, 7, 8), the incorporation of glutamate-C^{14} is markedly higher in a sucrose-phosphate homogenate (in which the integrity of the particulate material is maintained) than in homogenates prepared in phosphate or tris-(hydroxymethyl)-aminomethane (Tris) buffers. Significantly, the glycolytic system does not seem to be of great importance to amino acid incorporation in the cell-free preparations studied here. As is shown in table I the glycolytic intermediates, hexose diphosphate and 3-phosphoglycerate, do not enhance amino acid incorporation. Moreover, 0.01 M sodium fluoride, a potent glycolytic inhibitor, inhibits incorporation only

TABLE I

CHARACTERISTICS OF THE PROCESS OF AMINO ACID INCORPORATION INTO THE PROTEIN OF PEA SEEDLING HOMOGENATES

GRINDING MEDIUM	ADDITION TO SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
		$\mu M/hr \times gm\ protein$
0.1 M Potassium phosphate buffer (pH 7.5)	None	0.15
0.1 M Tris-HCl (pH 7.5) ..	None	0.10
0.45 M Sucrose + 0.05 M phosphate buffer (pH 7.5)	None	0.41
"	Hexose diphosphate	0.41
"	3-Phosphoglycerate	0.43
"	NaF	0.36
"	HCN	0.05
"	Hydrogen azide	0.08
"	Nitrogen atmosphere	0.20
"	CO (dark)	0.20
"	CO (light)	0.38
"	Dinitrophenol	0.19

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.001 M each of the inhibitors or other compounds. CO/O_2 ratio was approximately 19. Light intensity incident on the experimental vessels was about 300 fc.

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[2] Supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Company.

TABLE II

PARTICIPATION OF ATP IN THE INCORPORATION OF GLUTAMATE-C^{14} INTO PROTEIN

SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm$ protein
Untreated homogenate	0.43
" " + ATP	0.38
Dialyzed homogenate	0.16
" " + ATP	0.35
" " + ADP	0.10
" " + AMP	0.15

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), and 1.0 ml of either sucrose-phosphate homogenate or 1.0 ml of this homogenate after dialysis against sucrose-phosphate at 1° C. Where applicable, 0.001 M of the adenyl phosphates was present.

slightly. In contrast to this, the data of table I demonstrate a marked dependence of the incorporation process on oxidative activity. The incorporation is greatly inhibited by cyanide, azide, and by anaerobiosis. The marked inhibition of incorporation obtained with carbon monoxide is almost completely eliminated by light, which strongly indicates a participation of the oxidative system of peas in the incorporation process. The pronounced inhibition produced by dinitrophenol suggests, in agreement with previous results (11), that amino acid incorporation in these preparations is dependent on oxidative phosphorylation and, therefore, probably on ATP.

PARTICIPATION OF ATP IN AMINO ACID INCORPORATION: Despite the above evidence for the participation of oxidative phosphorylation (and, therefore, probably ATP) in the incorporation process, it is not possible to demonstrate a necessity for ATP unless the cell-free extracts are dialyzed (table II). The strong inhibition of glutamate incorporation obtained on dialysis can be largely restored by the addition of ATP. As is evident from table II, adenosinemonophosphate (AMP) is ineffective in restoring the activity while adenosinediphosphate (ADP) is somewhat inhibitory. These results agree both with those previously obtained with plant mitochondrial preparations (11) and with mammalian particles (16). It seems quite likely that ATP is indeed necessary for at least a portion of amino acid incorporation.

TABLE III

EFFECT OF DIVALENT IONS ON THE INCORPORATION OF GLUTAMATE-C^{14} INTO PEA SEEDLING HOMOGENATES

DIVALENT ION ADDED TO SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm$ protein
None	0.43
Mg	0.67
Mn	0.46
Ca	0.30
Co	0.29
Zn	0.06
Cu	0.03
Ba	0.01

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.01 M of each of the metal ions listed, and 1.0 ml of sucrose-phosphate homogenate in a total volume of 2 ml.

EFFECT OF DIVALENT IONS ON INCORPORATION: The apparent participation of ATP in amino acid incorporation (presumably by some transphosphorylation reaction) suggests that magnesium ions might also be required for this process as they are for amide (10) and peptide (13, 14, 15) syntheses, and in fact for most reactions involving ATP. As is evident from table III, of a number of divalent ions examined, only magnesium ions produce a significant increase in the rate of glutamate-C^{14} incorporation. Most other divalent ions are inhibitory. It is noteworthy that the effects of the different divalent ions on glutamate incorporation are qualitatively very similar to their effects on the biosynthesis of simple peptides (14).

INCORPORATION OF AMINO ACIDS OTHER THAN GLUTAMATE: The data of table IV show that incorporation by tissue homogenates is not confined to glutamate, but proceeds at easily measurable rates with each of a number of C^{14}-labeled amino acids and in cell-free extracts of three different tissues. It is noteworthy that the rates of incorporation of the various amino acids differ markedly in the same tissue. Furthermore, the rate of incorporation of any one amino acid is, in general, different in the different tissue extracts. It is not clear at present whether these differences reflect: (a) a requirement for varying amounts of the different amino acids for protein formation, (b) different rates of exchange of the amino acids into preformed protein, or (c) differences in the rates of breakdown of the different amino acids. It is possible that all three processes are involved.

EFFECTS OF OTHER AMINO ACIDS ON THE INCORPORATION OF GLUTAMATE-C^{14}: As is evident from table V, the addition of various individual amino acids to the system generally has no marked effect on the rate of glutamate incorporation. In a few cases, however, inhibition of glutamate incorporation has been observed. Such inhibition occurs in the pres-

TABLE IV

INCORPORATION OF RADIOACTIVE AMINO ACIDS BY PLANT HOMOGENATES

AMINO ACID	μM AMINO ACID INCORPORATED/HR × GM PROTEIN		
	PEA SEEDLINGS	BEAN SEEDLINGS	WHEAT SEEDLINGS
Glutamate-2-C^{14}	0.43	0.35	0.50
Aspartate-4-C^{14}	0.14	0.21	0.15
Glycine-2-C^{14}	0.59	0.37	0.48
Cysteine-S^{35}	0.36	0.43	0.21
Histidine-1-C^{14}	0.47	0.16	0.28
Leucine-2-C^{14}	0.58	0.69	0.48
Serine-1-C^{14}	0.48	0.40	0.21
Alanine-1-C^{14}	0.40	0.36	0.33

ence of aspartate, asparagine, and citrulline. It is possible that the inhibition may be due to a similarity in structure of these compounds to glutamate, a similarity which allows them to compete with glutamate for sites in the incorporating system. Especially interesting is the inhibitory action of citrulline while the analogous arginine has no effect on incorporation. Likewise, α-aminobutyrate inhibits glutamate incorporation, but γ-aminobutyrate is without effect.

The incorporation of glutamate is markedly increased by the simultaneous addition of a mixture of 17 amino acids to the system. The cause of this increase is not as yet clear. It is well known that protein synthesis in intact cells occurs only if all of the component amino acids are simultaneously present. It cannot be concluded from the present data that the enhanced incorporation of glutamate in the presence of the 17 other amino acids is a reflection of protein synthesis by the system, as no evidence for actual protein synthesis is available. Similar results, however, have been obtained by Gale and Folkes (3, 4) with bacterial breis in which apparent enzyme synthesis occurs only in the presence of an amino acid mixture similar to that used here.

In order to investigate further the promotion of incorporation by the mixture of amino acids, the ability of various antagonists of amino acids other than glutamate to inhibit the enhanced glutamate incorporation has been examined. That such antagonists are highly effective is illustrated by the data of table VI. Both *p*-fluorophenylalanine and β-2-thienylalanine, antagonists of phenylalanine, are strong inhibitors of glutamate incorporation in the presence of the mixture of 17 other amino acids. Likewise, significant inhibitions are obtained with ethionine (an antagonist of methionine) and with allyl glycine (an antagonist of cysteine). These results suggest that the enhanced incorporation of radioactive glutamate may be the result of some process in which all of the amino acids are involved.

TABLE V

EFFECT OF VARIOUS AMINO ACIDS ON THE INCORPORATION OF GLUTAMATE-C^{14} INTO THE PROTEIN OF PEA SEEDLING HOMOGENATES

AMINO ACIDS ADDED *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm$ protein
None	0.43
α-Aminobutyrate	0.38
Aspartate	0.24
Asparagine	0.33
Citrulline	0.30
Individual amino acids **	0.43
Mixture of 17 amino acids	0.71

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.01 M each of the amino acids or 0.2 ml of the mixture of 17 amino acids and 1 ml of homogenate in a total volume of 2 ml. Incubation was for 5 hrs at 38° C.

** The following individual amino acids were without effect on glutamate-C^{14} incorporation when incubated with the above system: α-alanine, β-alanine, γ-aminobutyric acid, arginine, cysteine, dihydroxyphenylalanine, glycine, histidine, leucine, lysine, methionine, phenylalanine, proline, serine, tryptophane, tyrosine (0.0001 M), and valine.

TABLE VI

EFFECT OF AMINO ACID ANALOGUES ON INCORPORATION OF GLUTAMATE-C^{14} INTO PROTEIN IN THE PRESENCE OF A COMPLETE AMINO ACID MIXTURE

ADDITION TO SYSTEM *	GLUTAMATE-C^{14} INCORPORATED
	$\mu M/hr \times gm$ protein
None	0.71
p-Fluorophenylalanine	0.47
β-2-Thienylalanine	0.40
Ethionine	0.61
Allylglycine	0.58

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.01 M of each of the amino acid antagonists, 0.2 ml of the mixture of 17 amino acids, and 1 ml of homogenate in a total volume of 2 ml. Incubation was for 5 hrs at 38° C.

INCORPORATION BY VARIOUS CELLULAR FRACTIONS: It was reported earlier (12) that when bean seedlings are incubated with a radioactive amino acid, the small particle (possibly microsomal) fraction incorporated the greatest amount of radioactivity. The data of table VII show that intact tissues of bean and pea seedlings possess similar patterns of glutamate incorporation into their various cellular fractions. Furthermore, in both cases cell-free extracts prepared from the seedlings incorporated glutamate in a manner analogous to that of the intact seedlings. It

TABLE VII

INCORPORATION OF GLUTAMATE-C^{14} INTO PROTEIN BY INTACT SEEDLINGS, BY HOMOGENATES, AND BY ISOLATED CELLULAR FRACTIONS

CELLULAR FRACTION *	INTACT TISSUES	HOMOGENATE	ISOLATED PARTICLES
		$\mu M/hr \times gm$ protein	
Pea seedling:			
Nuclear	0.41	0.48	0.13
Mitochondrial	0.68	0.63	0.31
Microsomal	1.05	1.00	0.05
Soluble	0.29	0.20	0.15
Bean seedling:			
Nuclear	0.15	0.18	0.11
Mitochondrial	0.43	0.55	0.38
Microsomal	1.18	1.83	0.09
Soluble	0.31	0.59	0.25

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), and the indicated tissue preparations. Incubation was at 38° C. The nuclear fraction includes all material sedimenting at 500 × g for 5 min. The mitochondrial fraction includes all material sedimenting between 500 and 10,000 × g for 10 min, while the microsomal fraction encompasses that material sedimenting between 10,000 and 100,000 × g for 30 min. That protein not sedimenting at 100,000 × g is designated as soluble.

should be emphasized here that the designations of the cellular fractions are entirely arbitrary and do not represent homogeneous preparations of these fractions. If the various cellular fractions are incubated separately with glutamate-C^{14}, the specific activity of every fraction is considerably reduced. The greatest reduction occurs in the case of the small particle fraction. It seems likely that one or more of the other fractions are necessary for the relatively large amount of incorporation that occurs in the small particle fraction of the whole homogenate. In order to test this, the incorporating ability of various combinations of cellular fractions has been examined. Table VIII shows that only a few of such combinations result in relatively high incorporation rates. It is noteworthy that in each of these cases the mitochondrial fraction constitutes one component of the system. In accord with results from intact cells and whole homogenates, a system composed of the mitochondrial and microsomal fractions exhibits by far the greatest specific activity. This finding is similar to the results of Siekevitz (9) and of Keller et al (5) with cellular fractions of mammalian tissues. It would appear, therefore, that an experimental system composed of these two cellular fractions offers the most logical starting place for studies on the nature of the process of amino acid incorporation and its relation to protein synthesis. The system may be still further simplified by fractionation of the microsomal material (table IX). The particulate material which sediments at $40{,}000 \times g$ has the ability (when mixed with the mitochondrial fraction) to carry out incorporation of glutamate-C^{14} at the highest rates thus far observed during these studies, while the particulate material which sediments between $40{,}000 \times g$ and $100{,}000 \times g$ has a low activity more comparable to that of the soluble fraction. Apparently only a certain type or size range of particles is involved in the very active incorporation reported here.

Effect of Supplemental Amino Acid Mixture on Differential Incorporation by Cellular Fractions: The different rates of glutamate-C^{14} incorporation by the cellular fractions have been compared in the presence and absence of the supplemental mixture of 17 amino acids (table X). It is evident that glutamate-C^{14} incorporation by the microsomal fraction is markedly enhanced by the presence of the other amino acids. The same is true, to a lesser extent, of the mitochondrial fraction. In contrast, the soluble fraction shows a marked decrease in specific activity while the nuclear fraction is little affected. These findings support the previously stated suggestion that a particulate system composed of the mitochondrial and microsomal fractions would seem to offer the most promising site for further investigations of the nature of amino acid incorporation into cellular protein in plants.

Table VIII

Incorporation of Glutamate-C^{14} by Various Combinations of Cellular Fractions of Pea Seedlings

System *	Glutamate-C^{14} incorporated
	$\mu M/hr \times gm$ protein
Total homogenate	0.39
Homogenate – nuclear fraction	0.66
Nuclear + mitochondrial fractions	0.88
Nuclear + microsomal "	0.23
Nuclear + soluble "	0.40
Mitochondrial + microsomal "	1.28
Mitochondrial + soluble "	0.65
Microsomal + soluble "	0.20

* Complete system contained: 0.01 M glutamate-C^{14}, 0.05 M potassium phosphate buffer (pH 7.5), 0.001 M ATP and 1.0 ml of cellular fraction in a total volume of 2 ml. Incubated at 38° C for 3 hrs.

Table IX

Incorporation of Glutamate-C^{14} by Particulate Fractions

System *	Glutamate-C^{14} incorporated
	$\mu M/hr \times gm$ protein
Mitochondrial + particulate fraction sedimented between:	
10,000 and $100{,}000 \times g$	1.28
10,000 and $40{,}000 \times g$	1.50
40,000 and $100{,}000 \times g$	0.73

* System contained the same components as described under table VIII.

Table X

Effect of the Presence of a Supplemental Amino Acid Mixture on the Distribution of Incorporated Glutamate-C^{14} in Pea Seedling Homogenates

Fraction *	Glutamate-C^{14} incorporated	
	Glutamate alone	Glutamate + 17 amino acids
	$\mu M/hr \times gm$ protein	
Nuclear	0.48	0.46
Mitochondrial	0.63	0.72
Microsomal	1.00	1.25
Soluble	0.20	0.16

* Composition of system and incubation time were the same as described under table VIII.

Discussion

Two facts of potential importance have emerged from the present investigation. It has been shown that a relatively simple cell-free system which carries on the incorporation of amino acids into protein may be prepared. Like the organized respiratory systems that have been studied so extensively in both plants and animals, the activity of this system is dependent on its method of preparation. Furthermore, like various other systems which carry out biological syntheses, the amino acid incorporation system apparently involves ATP. The active incorporation system

described here may be treated operationally just as any organized complex of enzymes (such as those concerned with pyruvate oxidation or with fatty acid metabolism).

The second important finding is that the incorporation of one amino acid may be promoted by the presence of a mixture of the other amino acids normally found in protein. While it is premature to suggest that protein formation may occur in the particulate system in the presence of the amino acid mixture and a suitable energy source, the effect deserves further investigation.

Summary

The incorporation of various amino acids into the proteins of plant homogenates has been demonstrated. Incorporation rates vary greatly according to the method of preparation of the homogenate. The process is dependent on ATP and is considerably enhanced by magnesium ions, but is inhibited by most other divalent ions. Incorporation of glutamate generally is unaffected by the presence of other individual amino acids, but is inhibited by aspartate, asparagine, and citrulline. However, a mixture of 17 amino acids greatly increases glutamate incorporation. This enhanced incorporation of glutamate is strongly inhibited by antagonists of various amino acids. A particulate fraction of cellular homogenates of pea seedlings has been prepared which incorporates amino acids at high rates.

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NONVOLATILE ORGANIC ACIDS OF CROWN GALLS, CROWN GALL TISSUE CULTURES AND NORMAL STEM TISSUE[1,2]

R. W. SCOTT, R. H. BURRIS and A. J. RIKER

Departments of Biochemistry and Plant Pathology, University of Wisconsin, Madison 6, Wisconsin

Measurements of the organic acids of galls, stems, and gall tissue cultures have been undertaken. Comparisons among these plant tissues are of interest because the tissues have like origins but unlike differentiation. The changes are obvious in the gross appearance of crown galls, which develop at stem wounds infected with *Agrobacterium tumefaciens*. These galls far outgrow the surrounding stem tissues and become increasingly disorganized. Crown gall tissues of several plants have been obtained bacteria-free and have been grown in sterile cultures (6). In contrast to cultures of normal stem tissues, the gall tissues grow in a simplified medium and they do not differentiate to form roots or other organs. Detailed comparisons of normal stems and crown gall tissue

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growing both in vivo and in vitro may yield useful information concerning the interesting changes when normal growth becomes abnormal and concerning chemical changes in tissue removed from in vivo to in vitro culture.

The organic acids of normal and gall tissues were compared because they are an important group of metabolites. Hildebrandt et al (6, 7) and Riker and Gutsche (14) found that certain concentrations of some amino acids and nonnitrogenous organic acids strongly inhibited the growth of crown gall tissue cultures; a few acids were slightly stimulatory. Eberts et al (3) have found active glutamic acid–aspartic acid and glutamic acid–alanine transaminases in the same stocks of sunflower crown gall tissue cultures as were used in the present work. This has some bearing on the following experiments because α-ketoglutaric acid was here found to be a major organic acid in both sunflower and periwinkle tissue cultures.

Materials and Methods

Tissues: Plants used for analyses were sunflower, *Helianthus annuus* L., var. Giant Russian; marigold, *Tagetes erecta* L., var. Sunset Giant; periwinkle, *Vinca rosea* L., and tomato, *Lycopersicon esculentum* Mill. var. Bonny Best. In all experiments but one indicated in table I the plants were grown in a greenhouse. Normal stem tissue was taken from the top one or two developing internodes of uninoculated plants unless designated as mature stem tissue. In the latter case it was taken from mature internodes of uninoculated plants. The crown gall tissue was produced by inoculating the listed plants with *Agrobacterium tumefaciens* (Smith and Townsend; Bergey et al). This gall tissue was separated from the stem as much as possible. Small galls of sunflower and tomato were 3 to 8 mm in diameter when harvested 2 or 3 weeks after the inoculations were made. Marigold, and especially periwinkle galls took 2 to 4 weeks longer for comparable growth.

The crown gall tissue cultures were obtained from stocks cultivated by Hildebrandt et al (4, 6). All tissue cultures were grown on a modification of Hildebrandt's improved sunflower tissue medium (5). The stock solutions together with the concentrations of salts in the medium after mixing were as follows:

Salts	Gm/liter in the stock solution	Mg/liter in the medium after mixing
Solution A		
Na_2SO_4	20	100
$MgSO_4 \cdot 7\,H_2O$	144	720
KCl	26	130
$MnSO_4 \cdot 4\,H_2O$	0.9	4.5
$ZnSO_4 \cdot 7\,H_2O$	0.6	3.0
H_3BO_3	0.6	3.0
Solution B		
$Ca(NO_3)_2 \cdot 4\,H_2O$	160	800
KNO_3	32	160
Solution C		
$NaH_2PO_4 \cdot H_2O$	132	132
Solution D		
$Fe_2(SO_4)_3$ + Aq.	5	5

The medium was prepared from the refrigerated stock solutions by adding 5 ml of A, 5 ml of B, 1 ml of C and 1 ml of D to 990 ml of distilled water. To prevent precipitation of iron compounds, solution D was kept acidified with a small quantity of sulfuric acid and was the first stock solution diluted. The complete medium was adjusted to pH 5.5 to 6.0 with three drops of 5 *N* KOH, and 2 % of reagent-grade sucrose was added.

Marigold crown gall tissue cultures were grown on the above medium plus 0.6 % agar. They were analyzed for organic acids after three weeks on fresh medium. Sunflower and periwinkle crown gall tissue cultures were grown on the medium without agar. The latter cultures were kept in about 25 ml of me-

Table I

Dry and Wet Weights and Total Titration * of Nonvolatile Acids of Various Sunflower Tissues

Sunflower tissue	N/gm dry wt	N/gm fresh wt	$\frac{\text{Dry wt}}{\text{Fresh wt}} \times 100$	Total acid: per gm N	Total acid: per gm dry wt **	Total acid: per gm fresh wt
	mg	*mg*		*meq*	*meq*	*meq*
a. Rapidly growing crown gall tissue cultures	73.5	0.71	1.0	53	3.9	0.038
b. Rapidly growing top internodes—exp. 1	63.9	0.80	1.3	55	3.5	0.044
c. Rapidly growing top internodes—exp. 2	57.4	0.81	1.4	40	2.3	0.032
d. Young galls on greenhouse plants—exp. 1 (3–8 mm in diameter)	39.9	1.71	4.3	30	1.5	0.065
e. Old galls on greenhouse plants—exp. 3 (10–25 mm in diameter)	9.4	1.42	15.1	87	0.8	0.123
f. Stem tissue adjacent to above old galls—exp. 3	5.7	0.63	11.1	234	1.3	0.148
g. Stem tissue of large field plants in flower	10.9	1.15	10.5	...	..	....

* Sum of the analyzed nonvolatile acids.
** The dry wt of the pressed cake; i.e., wt of water-insoluble material.

dium in 125-ml Erlenmeyer flasks on a reciprocating shaker with a 6-inch stroke and 1-second cycle. Periwinkle tissue was transferred from agar cultures to the liquid medium, allowed to grow for 2 to 3 weeks and transferred to fresh liquid medium a week before harvesting. Sunflower crown gall tissue cultures have been maintained for four years on the liquid medium. When transferred to fresh medium every 2 or 3 weeks, the cultures continued a uniformly vigorous growth, which was about 0.1 gm per gram of fresh tissue per day at 26° C. It should be noted that this continuous growth was maintained in a medium devoid of any added growth substances. The fresh weight of the pieces varied from 0.25 to 1.0 gm when the cultures were transferred.

Extraction of Nonvolatile Acids: Five to 20 gm of fresh tissue were ground with 100 ml of water in a Waring Blendor and the liquid was expressed through felt pads on a Carver press under a pressure of about 1500 lbs/inch^2. Twenty-five ml of 0.5 *N* sulfuric acid was mixed with the residue in the cylinder and pressed out. The pressed residue was transferred to a small beaker containing 25 ml of 0.5 *N* sulfuric acid and briefly warmed almost to boiling. The mixture was cooled and pressed. A final rinse of 25 ml of water was stirred with the residue and pressed out.

The combined liquid extract was concentrated to about 15 ml by vacuum distillation. An equal volume of acetone was added, and the precipitate was centrifuged down and rinsed twice with 25 ml of 50 % acetone. This precipitate was saved for nitrogen analysis. The acetone was removed under reduced pressure, and the remaining water solution was transferred to a Kutscher-Steudel extractor; the final volume was kept less than 30 ml. Freshly distilled ether was used. After a 3-day extraction, 1 or 2 ml of water was added to the extract and the ether was distilled. A small amount of fatty substance in the extract was removed with 5 to 10 ml of petroleum ether. The petroleum ether was back washed with a little water and the washings were returned to the extract. The small volume of extract was transferred to a 15-ml graduated centrifuge tube and evaporated down to 2 or 3 ml by directing an air stream over the liquid while warming. A 0.1- to 0.3-ml aliquot was dried onto a 0.03-inch-thick filter paper disk and chromatographed on a silicic acid column by successive changes of solvent (15). The elution chromatogram of acids from sunflower tissue cultures is shown in figure 1. After elution and titration, the acid peaks were tested by paper chromatography as described in the above reference. When paper chromatography clearly showed more than one component in a single peak eluted from a column, the relative amounts of the components were estimated by the intensity of their spots on the paper chromatogram.

The dry weight of tissue was taken as the weight of the pressed cake dried at 100° C. This excluded the weight of soluble material, which actually constituted a few percent of the total dry weight.

Nitrogen analyses were made by a semi-micro

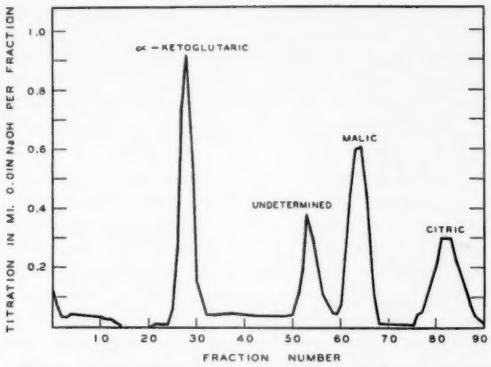

Fig. 1. The elution chromatogram of the nonvolatile organic acids extracted from sunflower crown gall tissue cultures.

Kjeldahl method (8, 17) on aliquots from the dried, pressed cake and on aliquots of a slurry made from the material precipitated with acetone and the aqueous solution remaining after ether extraction. The designated total nitrogen was calculated from the analyses of the aliquots.

Results and Discussion

Extraction and Identification of Acids: Pucher et al (13) have reported good recoveries of citric, isocitric, malic, oxalic and succinic acids from dried plant tissue. The methods described herein were even milder than the drying procedures and should not cause destruction of common plant acids. The results of Krebs and Eggleston (10) suggest that any aconitic acid would be found mostly in the *trans* form because of the low pH during extraction of our tissues.

The efficiency of the extraction was checked by re-extracting, together, several batches of the dried residues. No significant amount of titratable acids was found. Recoveries of standards from the chromatograph columns were between 90 and 100 % except that fumaric acid recovery varied down to as low as 75 %. This was later found to be a result of drying the sample on the filter paper pad. Excellent recoveries of fumaric acid result from introducing it as a solution in 0.5 *N* H_2SO_4 as in the method described by Zbinovsky and Burris (20).

Identification of most of the acids depended upon comparing their movement with that of standards on a silicic acid column and on two paper chromatograms developed with differing solvents. Citric and malic acids had previously been found in sunflower gall tissue cultures by chemical analysis and along with oxalic acid were to be expected in plant tissues. The large amount of α-ketoglutaric acid found in sunflower tissue cultures was unexpected, so the identity of this acid was further verified. A 1-gm silicic acid column was prepared with an internal phase of 0.5 ml of 7 % potassium phosphate buffer at pH 3.0. When eluted with 20 % 4-butanone in 4-methyl-2-

pentanone, succinic acid passed through the column before α-ketoglutaric acid (15). About 6 % of the total amount of acid in the large peak labelled α-ketoglutaric in figure 1 separated on the buffered column as succinic acid. About 4 meq of the acid in the second peak was recovered from a similar but larger buffered column. The 2,4-dinitrophenylhydrazone was prepared and recrystallized from ethyl alcohol-petroleum ether (78° C). It decomposed at 213 to 214° C, which was 9° lower than reported by Krebs and Johnson (9) but agreed with the decomposition point of the 2,4-dinitrophenylhydrazone prepared from commercial α-ketoglutaric acid (Nutritional Biochemicals Corporation, Cleveland, Ohio). Furthermore, these hydrazones chromatographed similarly on a silicic acid column (1) and had identical absorption peaks near 380 mμ in 10 % Na_2CO_3 and near 430 mμ in 1.2 *N* NaOH.

The identity of fumaric acid was verified by using its well known transformation to maleic acid when its alcoholic solution is exposed to ultraviolet light. Microgram amounts of fumaric acid were irradiated for one hour before spotting on paper chromatograms. The developed papers showed spots for both fumaric and maleic acids.

The acid which was eluted from the column at about 50 (fig 1) was first thought to be glycolic acid, for authentic glycolic acid was eluted there. However, when this acid was run on paper chromatograms, it had an R_f much lower than the R_f of authentic glycolic acid. This unidentified acid was found in all the plant tissues tested and was a major constituent of the acids of growing stems of sunflower and marigold. Minor amounts of still other unidentified acids were found in several tissues by paper chromatography. By cautiously avoiding heat during the preparation of the sample of young sunflower stems, a large amount of labile acidity was found in a very broad peak between succinic and malic acids. This fraction was not identified and the results in

TABLE II

NONVOLATILE ACIDS OF STEMS, GALLS AND TISSUE CULTURES OF GALLS

TISSUES	ACIDS								
	FUMARIC	LACTIC *	SUCCINIC *	α-KETO-GLUTARIC *	OXALIC	UNDETER-MINED **	MALIC	CITRIC-ISOCITRIC	TOTAL IN TABLE
	Meq/gm N								
Marigold									
Stem	28.5	1.8	1.9	...	...	14.3	3.7	2.3	52.5
Gall	33.6	...	2.3	...	5.8	8.3	14.3	9.4	73.7
Culture	2.0	0.9	0.9	...	...	4.9	4.7	10.0	23.4
Periwinkle									
Stem	...	...	19.5	...	...	3.3	22.6	24.4	69.8
Gall	0.9	...	6.8	...	...	2.4	7.5	22.7	40.3
Culture	1.5	0.5	0.5	18.1	...	6.7	20.8	3.0	51.1
Sunflower									
Mature stem	27.4	...	9.1	3.0	41.7	6.5	45.1	67.3	200.1
Growing stem	6.2	...	1.2	...	2.5	3.0	3.6	1.5	18.0
Old galls	19.8	...	2.4	5.1	17.9	4.8	31.8	10.0	91.8
Young galls	8.2	0.3	2.7	...	4.5	5.3	11.0	3.2	35.2
Culture	...	...	1.1	13.3	...	7.1	14.4	11.0	46.9
Tomato									
Stem	...	1.0	1.4	...	27.4	4.4	61.5	3.0	94.3
Gall	...	...	0.8	0.4	12.6	2.3	37.6	4.9	56.3
	% meq in elution chromatogram								
Marigold									
Stem	48.5	3.0	3.2	...	...	24.4	6.4	3.9	89.4
Gall	44.2	...	3.0	...	...	10.9	18.9	12.3	89.3
Culture	7.1	3.2	3.2	trace	...	17.8	17.0	36.3	84.6
Periwinkle									
Stem	...	...	24.6	...	...	6.3	29.0	31.4	91.3
Gall	1.9	...	15.0	trace	...	5.4	16.6	50.4	89.0
Culture	2.7	0.9	0.9	34.0	...	12.7	39.0	5.6	95.8
Sunflower									
Mature stem	11.7	...	3.8	1.2	17.7	2.7	19.3	28.8	85.2
Growing stem	30.5	trace	5.7	...	12.2	15.1	17.8	7.2	88.5
Old galls	20.4	trace	2.4	5.3	18.5	4.9	32.8	10.3	94.6
Young galls	27.0	1.0	9.0	trace	11.8	13.9	28.9	8.4	100.0
Culture	...	...	2.0	25.0	...	18.4	27.0	20.7	93.1
Tomato									
Stem	trace	1.0	1.4	...	27.2	4.4	61.0	2.9	97.9
Gall	trace	trace	1.4	0.7	21.3	3.9	63.5	8.2	99.0

* Where lactic, succinic and α-ketoglutaric acids did not appear singly, their proportions were estimated from paper chromatograms.

** Due only to titration of an acid or acids eluted from the columns just after oxalic acid.

tables I and II were obtained after the labile acidity was lost during the drying of the sample. Some of the other tissues may have contained similar labile acidity which was lost during analysis. Citric and isocitric acids were not distinguished from each other by the methods used. In table II, the last column in the second part gives the total percent of the acids identified plus the single unidentified peak after oxalic acid. Because of the probable losses of fumaric acid and small losses of other acids during chromatography the total percent is uncertain by several percent and probably is low.

Significance of Results: One of the variables involved in these experiments was the age of the tissue. Young developing internodes were usually 4 to 10 days old depending on the species, and mature internodes were usually 2 to 4 weeks older. The data of several experiments showed certain variations in composition of sunflower tissues as shown in table I. It is evident that percent dry weight increased with age (compare dry weight to wet weight ratio, lines b and c with line f, and compare lines d and e). With aging of the tissues the total measured acidity increased when based on wet weight or total nitrogen but decreased when based on dry weight. A few weeks were required for galls to develop, and the adjacent normal stem changed during this time as indicated in table I. The gall tissue analyzed could not be separated entirely from adjacent normal stem tissue so analyses of such gall tissue should be considered as resulting from gall tissue plus an indefinite but generally small amount of mature normal stem.

Gall tissue is characterized by continuous growth, so it might be argued logically that galls are best compared with young growing tissue. Nagy, Riker and Peterson (11) concluded that galls of tomato were like young plant tissues in their content of ash and nitrogen. The nitrogen and dry weight data in table I also indicate that the galls resembled young tissues. Some differences can be ascribed to dilution of gall tissue with unseparated mature stem tissue. In old galls the dry weight increased considerably because of the formation of sclariform cells. The total amounts of acids listed in table I show a rough similarity between galls and young plant tissues especially when expressed per unit nitrogen content. It is interesting to note the close agreement between the total amounts of acids of top internodes and crown gall tissue cultures.

Distribution of Acids: A comparison of the amounts of various acids listed in table II shows more striking differences than similarities among the tissues. No consistent and distinct acid pattern was found to be peculiar to gall tissue. Increases in citric, malic, and oxalic acids appeared to account for the increase in total acids as the tissue aged. There was a rough similarity between the major acids of the stems and galls in marigold, sunflower and tomato.

Tissue cultures had the most distinctive acid distributions. Marigold had considerably less fumaric acid in tissue cultures than in stems or galls. In periwinkle and sunflower tissue cultures the appearance of large amounts of α-ketoglutaric acid in the place of succinic and fumaric acids was striking. We are unaware of any other plant tissue containing such large amounts of α-ketoglutaric acid, although it has been found in a number of plants (18, 19). Since this acid was prominent only in the tissue cultures, its high concentration may have resulted from the conditions of culture. There might have been an inadequate supply of reduced nitrogen for reductive amination of the unoxidized α-ketoglutaric acid. The addition of a little ammonium nitrogen to the medium should have caused a rapid decrease in the α-ketoglutaric acid of the tissue, since Eberts et al (3) found active transaminases in sunflower tissue cultures. However, the content of α-ketoglutaric acid was not greatly reduced by inclusion of ammonium nitrogen in the medium. It may be significant that where α-ketoglutaric acid accumulated, a closely related acid of the normal stem (succinic acid of periwinkle and fumaric acid of sunflower) was not present in the tissue culture. This suggests that sunflower and periwinkle gall tissue cultures had sluggish α-ketoglutaric acid oxidase systems.

Fumaric acid was found in comparatively large amounts in marigold and sunflower stems, and it is possible that it may be more abundant and more widely distributed than previous analyses (12) have indicated.

Small amounts of callus tissue of normal sunflower stems only recently became available in this laboratory. This tissue has been grown on Hildebrandt's tobacco tissue medium (5) with 20 % of added coconut milk. Preliminary analyses for the acid distribution in stem callus cultures indicated certain resemblances to both gall tissue cultures and normal growing stems. However, the low level of α-ketoglutaric acid and the high level of succinic acid more nearly approximated the concentrations in normal stems. Approximate analyses were: malic 54.4 % of the total extracted acids, citric 19.9 %, succinic 12 %, α-ketoglutaric 4% , fumaric 2.0% , lactic 1.8 %, undetermined 1.7 %. The interesting difference between the amounts of succinic and α-ketoglutaric acids of normal and crown gall tissue cultures of sunflower may reflect either the abnormal nature of the gall tissue or merely the fact that these tissues were grown on different media.

Summary

1. Sunflower crown gall tissue cultures have been grown in a liquid medium containing reagent grade sucrose as the only added organic substance.

2. The individual organic acids from normal stems, crown galls and crown gall tissue cultures of marigold, periwinkle and sunflower, and from tomato stems and galls have been determined chromatographically.

3. Fumaric acid was a major constituent of the acids from marigold and sunflower stem tissues. An unidentified acid was abundant in the developing

stems of marigold and sunflower and smaller amounts were found in the other tissues analyzed.

4. Gall tissue and growing stem tissue of the same species did not differ appreciably in the kinds of organic acids they contained. Greater differences were found in the amounts of individual acids in normal and gall tissues. However, the differences which occurred in one species were not necessarily those found in another species.

5. Periwinkle and sunflower tissue cultures contained unusually large amounts of α-ketoglutaric acid. This acid was not a significant component of any of the other plant tissues.

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EFFECTS OF SPORULATION MEDIUM AND AGE ON FUNGUS SPORE PHYSIOLOGY[1]

RICHARD T. DARBY and G. R. MANDELS

Pioneering Research Division, Quartermaster Research & Development Center, Natick, Massachusetts

Basic physiological and biochemical data concerning fungus spores are sparse in relation to the importance of spores in biology, agriculture, and industry. One specific area of neglect has been the influence of the nature of the sporulation medium upon the subsequent behavior and metabolic activity of the spores. The only study directly concerned with this problem is that of Singh (9) whose work, along with certain other indirect data, is reviewed by Hawker (3). These older data relate only to effects upon rates of germination and are discussed by Hawker on the basis of accumulation in the spores of stimulatory growth substances from the medium.

The potential significance of the composition of the sporulation medium was forcefully brought to our attention by an experiment with *Myrothecium verrucaria*. Spores in cultures containing cellulose as carbon source were long-lived, whereas those in sucrose-

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yeast extract cultures suffered almost complete loss in viability within three weeks. The purpose of the present paper is to direct attention toward the marked influences which the cultural background of a spore may have on its physiology and biochemistry. To this end, results of exploratory studies relating to the respiratory activity, viability, and dry weight changes are reported for *M. verrucaria*. Some respiration experiments with *Memnoniella echinata* and *Aspergillus luchuensis* are included to demonstrate that qualitative as well as quantitative differences may occur.

Methods

The organism used for most of these studies was *Myrothecium verrucaria* (Alb. and Schw.) Ditm. ex Fr., (QM 460 = U.S.D.A. 1334.2). The substrates used to obtain spores, as well as the approximate yield of spores per Petri dish culture (9-cm dishes), are shown in table I. The basal salt solution contained in grams per liter 3.65 KH_2PO_4; 1.11 K_2HPO_4; 3.0 NH_4NO_3; 2.2 $MgSO_4 \cdot 7H_2O$; 15 agar (Bacto-Difco); pH 6.5. Different pH values were obtained by altering the phosphate ratio.

Plates, except those containing potato dextrose agar (PDA), were mass inoculated with 1 ml of an unwashed spore suspension in water containing 10^6 to 10^7 spores per ml, obtained from filter paper cultures. The PDA plates were inoculated with a special device which spaced individual colonies at about 7 mm intervals over the whole dish. This spacing was found to allow the individual colonies to reach an optimum size for sporulation. Cultures were grown and stored at 30° C. Under these conditions, sporulation is initiated within 2 to 3 days after inoculation and is completed within about 48 hours. Figures given as spore age are actually the age of the culture from which the spores were obtained. True spore age is thus about 3 days less.

Spores from filter paper cultures are readily brought into suspension by covering the culture with distilled water and shaking gently. Spores from other substrates and from old cultures were sometimes difficult to disperse and were loosened with a small brush; pieces of mycelium which sometimes came loose were removed by straining the suspension through several layers of cheesecloth. The freshly harvested spores were washed once with distilled water, once with buffer (0.05 M potassium phosphate, pH 6.2), and suspended in buffer. Aliquots of this suspension were used to determine dry weight, spore count, respiration, and nitrogen.

Substrates tested but abandoned because of low spore production included glycine, methionine, arginine, phenylalanine, acetate, citrate, egg albumin, yeast extract, α-amino butyric acid, casein, and casein hydrolysate. Some experiments were carried out using spores from sucrose, sucrose plus yeast extract (1 %), or glutamate media. The data are not reported since they did not reveal any responses significantly different from those obtained with the media reported here.

To obtain *Memnoniella echinata* (QM 1c) spores, the ammonium nitrate in the basal salts solution was reduced to 0.3 gm per liter and biotin was added to give 25 γ/l. Substrates used were filter paper, as for *M. verrucaria*, or 2 % sucrose. Culture vessels were 250-ml Erlenmeyer flasks containing 50 ml of medium. To prepare spore suspensions, 0.05 % Duponol was used as a wetting agent. Glass beads were added and the cultures shaken slowly to loosen the spores which were then washed twice as for *M. verrucaria*. Spore yields were of the order of 50 mg per culture from both substrates. *M. echinata* spores are borne in chains which cannot be broken readily, the suspensions thus being individual spores and chain fragments.

Spores of *Aspergillus luchuensis* (QM 873) were grown on the same basal salts solution as used for *M. verrucaria*, containing 2 % sucrose, or on PDA. Erlenmeyer flasks were employed as culture vessels and the same procedures for preparing spore suspensions were used as for *M. echinata*. Spore yields were about 25 mg per culture from both substrates.

Dry weights were determined with 5 to 10 mg of spores collected and washed on tared fritted glass crucibles (Corning "Fine") and dried overnight at 80° C. Spore counts were made with a Levy-Hauser counting chamber. Viability was determined by measuring percentage germination on PDA or by direct count of spore suspensions after short-time shaking in sucrose-yeast extract broth.

Nitrogen was determined colorimetrically after digestion and direct Nesslerization, according to the method of Miller and Miller (7).

Respiration was determined with conventional Warburg respirometers at 30° C with 1.0 ml of spore suspension containing 10^8 to 10^9 spores and 0.5 ml adjuvant in buffer in the side arm. Substrates were added to give 1 % final concentration; azide was used at 10^{-3} M. The Q_{O_2} (μl O_2 per mg dry wt per hr) values reported represent averages for the first 4 hours. The data for this time are not linear and, depending on substrate and spores used, may show increasing or decreasing slopes or sigmoidal curves (see 6, for example). Germination does not occur during the respiration runs with the substrates used.

Table I

Yield of M. verrucaria Spores from Different Sporulation Media *

Substrate	Initial pH	Spores per culture **
Cellulose (Whatman #1—7 cm filter paper disk)	6.5	5×10^9
0.5 % Peptone (Difco)	5.5	1×10^9
2.0 % Na succinate plus 0.1 % sucrose	5.5	1.6×10^9
Potato-Dextrose (Difco)	5.6	0.2×10^9

* All media contained basal salts.
** Median value of several counts.

Results

Filter Paper Spores—M. verrucaria: No loss in viability (fig 1) occurs up to about forty days. After this, there is a gradual decrease in viability to about 1 to 2 % at 100 days. Complete loss in viability of spore masses does not occur for at least several years, since subcultures can always be started from stock cultures of this age.

As the spores age there is a gradual, more or less linear, decrease in dry weight (fig 2). Some evidence based on photoelectric turbidity determinations of spore concentrations indicates that spore weight remains essentially constant for about the first three weeks (4). The reliability of this method for determination of concentration of spores of different ages is not known, since the optical properties may change during ageing.

Total nitrogen remains at about 9 to 12 % of the dry weight and appears to decrease at about the same rate as does the dry matter.

Respiration data (fig 3) show the endogenous respiration to be essentially constant at a Q_{O_2} of about 1 for spores up to 100 days old, although very young spores, immediately after formation, respire at a Q_{O_2} of about 4. Accurate measurement of endogenous respiration of older spores requires either larger quantities of spores or more sensitive apparatus than was used in this study. Endogenous respiration of *M. verrucaria* spores is initially high and decreases gradually to a constant value after 2 to 3 hours. This final level is about ⅓ of the initial rate, and the average rate for a period of 4 hours is about half that of the initial. Data (unpublished) show that the initial high rate is caused by the handling, centrifugation, and/or resuspension used to prepare the spores for study. In other words, the average endogenous values reported should not be given undue significance because of experimental errors and complex physiological factors involved.

Stimulation of endogenous respiration by azide is at a maximum at about 8 days and then declines gradually. Whether actual stimulation or inhibition occurs with 100-day-old spores has not been determined precisely.

Respiration on sucrose or peptone increases gradually to a spore age of about 50 days and then declines as viability starts to fall off. The activity on peptone is considerably higher than on sucrose. Although the points of the sucrose and peptone curves shown in figure 3 fluctuate widely, data from replicate experiments substantiate the shape of the curves drawn. Obviously, the extremely low Q_{O_2} in old spores is due largely to the considerable quantity of dead spores since the viability is only of the order of 1 % at 100 days. The Q_{O_2} in these latter stages is the resultant of several factors—the presence of dead material, the decrease in dry weight per spore, and, presumably, a decrease in the respiratory capacity of the old spores.

Peptone Spores—M. verrucaria: The viability of spores in peptone cultures declines very abruptly—less than 1 % viable spores remaining after about 12 days in one case and 26 days in another (fig 1). While the initial dry weight of peptone spores is about 75 % of that of filter paper spores, the rate of dry weight loss is approximately the same (fig 2). Nitrogen was from 9 to 15 % of the dry weight—varying from 165 $\gamma/10^8$ spores at 3 days to 240 at 7 days and 135 at 17 days. Respiratory activity of young spores is comparable with that of filter paper spores, but declines very rapidly—following the viability (fig 4). Some evidence indicates a peak in respiratory activity in very young spores attained within a day or two after formation.

Succinate Spores—M. verrucaria: Spores from succinate, like those grown in peptone, are very short-lived (fig 1). The spores have about the same initial dry weight as do filter paper spores, but the rate of loss in dry matter is much more rapid (fig 2). Nitrogen was from 10 to 14 % of the dry weight—varying from 200 $\gamma/10^8$ cells at 5 days to 265 at 10, 180 at 20, and 110 at 30 days. In contrast to the other types of spores, those from succinate respire sucrose and peptone at about the same rate (fig 5). Succinate spores are further characterized by a sharp maximum in respiratory activity at an age of about 10 days.

Potato Dextrose Agar Spores—M. verrucaria: PDA spores are considerably shorter-lived than filter paper spores, but the viability is somewhat better than for succinate or peptone spores (fig 1). The dry weight of very young spores is very high, around 40 to 45 mg/10^9 spores. However, this rapidly declines so that at 30 days it is less than 10 mg/10^9 spores (fig 2). The nitrogen content of PDA spores is very low—ca 2 % of the dry weight. This suggests that the high dry weight is due to non-nitrogenous reserves.

Respiratory activity of PDA spores (fig 6) on peptone is low—of about the same order as very young succinate spores. Respiration on sucrose is particularly low and decreases fairly rapidly to the approximate level of the endogenous rate. The azide effect is also relatively slight.

Memnoniella echinata Spores: Data on spore viability are not available, but the undiminished respiratory activity of both filter paper and sucrose spores from cultures 254 days old indicates them to be longer-lived than *M. verrucaria* spores. Of greatest interest is the differential effect of the inorganic constituents of the medium in the respirometer. Whereas respiration on glucose is markedly higher in basal salts than in buffer for both filter paper and sucrose spores, the endogenous respiration of sucrose spores is five times greater in basal salts (fig 7). Endogenous respiration of filter paper spores is not affected significantly by basal salts. With *M. verrucaria* spores, respiration is essentially the same in buffer and basal salts. Data (unpublished) indicate that biotin at 0.33 γ/ml increases respiration slightly.

Aspergillus luchuensis Spores: Although only respiratory data are available, spore longevity appears to be much greater for PDA spores than for sucrose spores. Spores from PDA cultures 198 days old respired glucose at essentially the same rate as

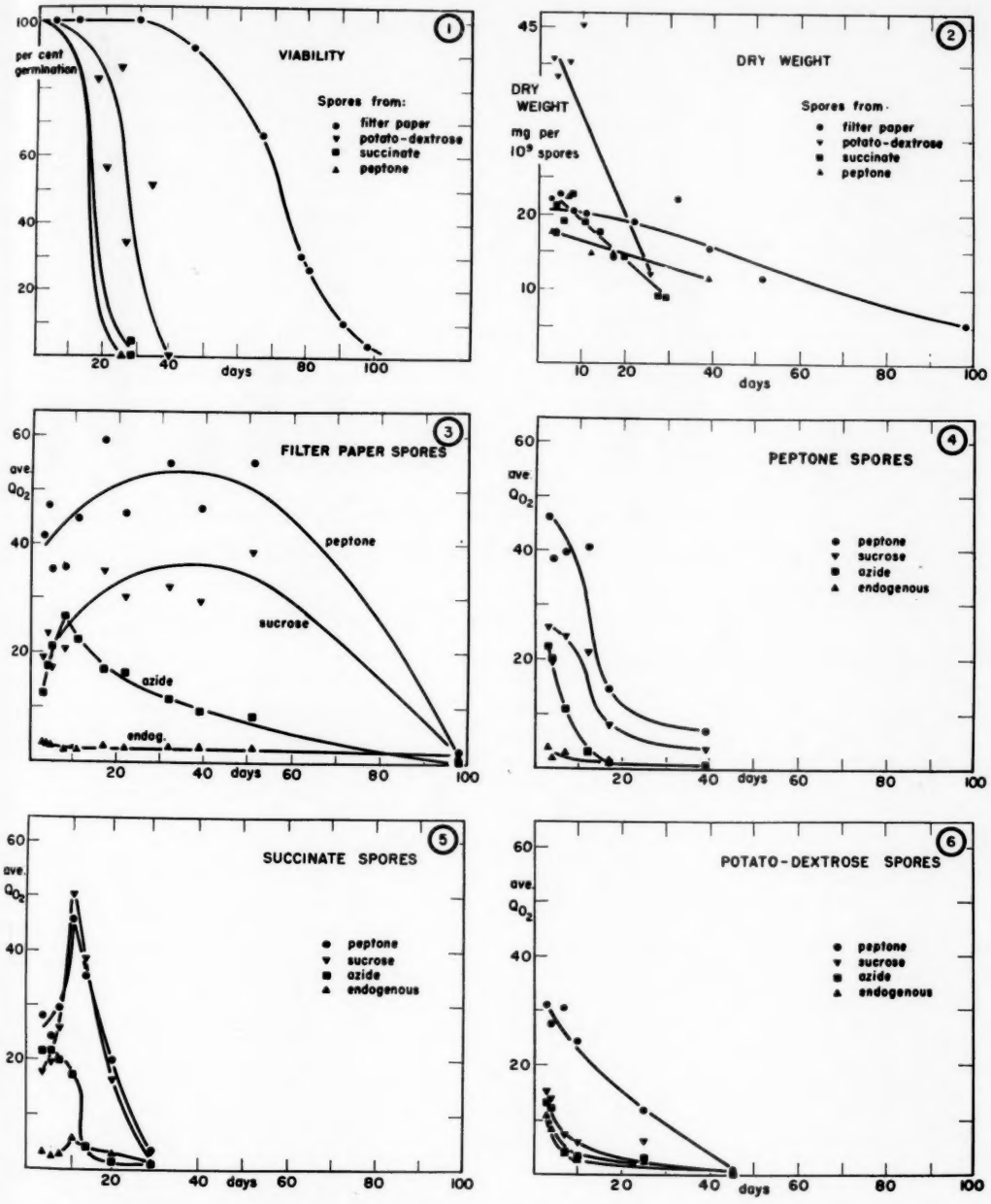

FIG. 1. Effect of source and age on viability of *M. verrucaria* spores.
FIG. 2. Effect of source and age on dry weight changes of *M. verrucaria* spores.
FIG. 3. Effect of age on respiratory behavior of *M. verrucaria* spores from filter paper cultures.
FIG. 4. Effect of age on respiratory behavior of *M. verrucaria* spores from peptone cultures.
FIG. 5. Effect of age on respiratory behavior of *M. verrucaria* spores from succinate cultures.
FIG. 6. Effect of age on respiratory behavior of *M. verrucaria* spores from potato dextrose agar cultures.

Note: In figures 1 to 6 days refers to age of culture from which spores obtained; Q_{O_2} is μl O_2 per mg dry wt/hr, averaged for the first 4 hrs.

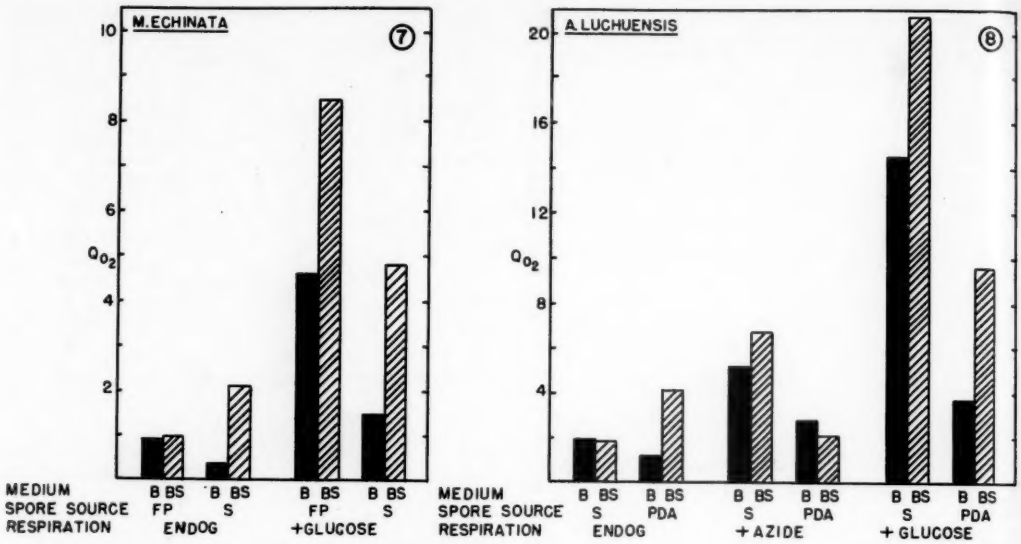

FIG. 7. Effect of buffer (B) vs. basal salts (BS) on respiration of *M. echinata* spores from filter paper (FP) or sucrose (S) cultures.

Average values from several experiments.

FIG. 8. Effect of buffer (B) vs. basal salts (BS) on respiration of *M. echinata* spores from sucrose (S) or potato dextrose agar (PDA) cultures.

Average values from several experiments.

young spores ($Q_{O_2} = 4.0$ and 7.5 for buffer and basal salts, respectively), while no measurable respiration was found for sucrose spores of the same age. Certain inorganic salts have a marked stimulatory effect on respiration of PDA spores, affecting both the endogenous and exogenous respiration (fig 8). Data not given here show this effect to be due to provision of the ammonium ion. Magnesium sulfate or potassium nitrate added to phosphate buffer do not stimulate respiration beyond that in buffer alone. While the endogenous respiration of sucrose spores is not influenced by addition of nutrient salts to the buffer, there is stimulation of oxygen uptake on glucose. Azide stimulates the endogenous respiration of sucrose spores markedly. With PDA spores, the effect of azide depends upon the composition of the medium. In phosphate buffer, the endogenous rate is doubled by azide, whereas in basal salts there is about 50 % inhibition. The much higher exogenous respiration of sucrose spores is noteworthy.

DISCUSSION

The great differences in longevity of spores obtained from various sporulation media indicate the importance of judicious selection of culture media for maintenance of stock cultures. Direct data establishing this point were obtained only for *M. verrucaria* spores. Indirect evidence (respiratory) with *A. luchuensis* spores suggests that this variable is of general significance. It should be noted that aging has occurred in the original cultures. The aging phenomenon in spores separated from stock cultures—either under lyophilization or other method of storage—has not been studied here and will not be considered.

In the initial stages of the investigation, it was thought that rapid loss in viability of some of the *M. verrucaria* spores might be ascribed to a high endogenous metabolism resulting in the consumption of reserves. This is not borne out by the dry weight nor by the endogenous respiration data. The longer-lived filter paper spores show a more gradual weight loss than the others, but there is not good correlation between rate of viability loss and rate of dry weight loss in various types of spores. The higher endogenous respiration of short-lived spores is not sufficiently greater than that of longer-lived ones to account for the decreased longevity.

The significance of the measurements of endogenous respiration with respect to their use in interpretations of senescence cannot be evaluated unambiguously, since the conditions in the respirometer are entirely different from those of the spores in cultures. Attempts were made, however, to quantitate the rate of dry weight loss of filter paper spores with the rate of oxidation of reserves, as calculated from the endogenous respiration. These show the endogenous value to be too great by a factor of about 10. Assuming the measured dry weight loss to be due entirely to oxidation of cellular constituents, the endogenous respiration of spores in the parent culture must be about 10 % that of the spores in the respirometers. Whether this is due to effects of moisture or removal

of inhibitory staling products present in the culture is not known. Experiments show, however, that the supernatant from the initial spore suspension has no inhibitory effects upon growth in sucrose-yeast extract culture (unpublished) or upon respiration (4). It is possible that dilution of inhibitory materials in the stock cultures prevented their detection. Thus, we can tentatively ascribe the increased endogenous respiration in respirometers to increased available water, although diffusion of oxygen and CO_2 may be an important limiting factor since the spores occur in dense masses in the culture. It can be assumed that the respiration of *M. verrucaria* spores in filter paper cultures is of the order of 0.5 μl per 10^8 cells per hour, or, on an initial dry weight basis, of a Q_{O_2} of about 0.2.

It is conceivable that old spores may fail to germinate simply because of additional requirements for germination or because of a secondary dormancy. This concept has been explored to some extent using different types of media and prolonged incubation (several weeks). All evidence suggested that if old spores do not germinate within a day they are dead. Previous studies (4) have shown that the requirements for germination of *M. verrucaria* spores change with age, older spores requiring either larger quantities or different kinds of extracellular substrates or growth factors.

The respiratory activity of *M. verrucaria* spores increases with age to a maximum, followed by a decline. These two stages can be considered as periods of maturation and senescence. The relative lengths of these stages are markedly influenced by the nature of the culture medium. Maturation extends over about 40 days in filter paper spores, whereas in succinate spores it lasts only about 11 days. With peptone and PDA spores maturation is so rapid that precise definition is not possible with the data available. Data not presented here show that in *M. echinata* spores maturation appears to be characterized by a decrease in respiratory activity, whereas in *A. luchuensis* the pattern appears more like that with *M. verrucaria*. Other types of maturation phenomena have been noted in *M. verrucaria* (5), in conidia of *Neurospore crassa* (8), and in ascospores of *N. tetrasperma* (1). Hawker (3) has reviewed other literature on the maturation of fungus spores as related to germination. Data relating to senescence of fungus spores has been reviewed by Gottlieb (2).

The effects of azide on spores of various ages and sources were studied because earlier data (Mandels, unpublished) had demonstrated marked stimulation of endogenous respiration of *M. verrucaria* filter paper spores. These data had also shown the magnitude of this stimulation to be influenced markedly by spore age—the effect being much less in old spores. Data in the present report were obtained in an attempt to determine this effect of age more precisely in view of the possibility of correlating the magnitude of the effect with vitality or with the amount of respirable reserves within the spores. The data obtained show the azide effect to be without value for either of these purposes, although marked differences are noted between spores from various sources.

All of the effects observed in *M. verrucaria* spores were quantitative in nature. With *A. luchuensis* and *M. echinata*, on the other hand, pronounced qualitative differences are found. Specifically, the existence of a stimulatory effect of basal salts on endogenous respiration depends upon the spore source.

It is commonly acknowledged that the composition of a growing organism can be markedly influenced by the nature of the environment, including the type of substrate upon which it has been growing. The significance of such environmental factors upon dormant structures such as spores has not been studied to a comparable extent. Data presented here demonstrate the profound influence that may be exerted upon spores by the type of culture medium. The effects upon viability, dry weight loss, and respiratory activity must reflect major modifications in spore composition, enzymic or otherwise. This is particularly evident from the effects of azide in producing stimulation or inhibition of the endogenous respiration of *A. luchuensis* spores depending upon the source of the spores and the nature of the inorganic constituents in the medium in the respirometers.

Summary

1. The nature of the medium upon which sporulation occurs has a profound influence on the physiology of spores of *Myrothecium verrucaria*, *Memnoniella echinata*, and *Aspergillus luchuensis*. Both qualitative and quantitative effects are found.

2. *M. verrucaria* spores from a cellulosic substrate are much longer-lived than those from succinate, peptone, potato dextrose, or sucrose plus yeast extract cultures. The rates of change in dry weight with age, as well as the initial dry weight of the spores, differ radically although there is no correlation with loss in viability. Nitrogen content varies between the extremes of 2 % of dry weight for spores from potato dextrose to 15 % for spores from peptone cultures. Respiratory activity on different substrates, as well as the stimulatory effects of azide on endogenous respiration of spores from these sources, differ greatly.

3. Basal salts markedly stimulate endogenous respiration of *M. echinata* and *A. luchuensis* spores from certain media. Azide either stimulates or inhibits endogenous respiration of *A. luchuensis* spores, depending upon the spore source and salt composition of the suspending medium.

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STUDIES ON NITROGEN-FIXING BLUE-GREEN ALGAE. I. GROWTH AND NITROGEN FIXATION BY ANABAENA CYLINDRICA LEMM.[1]

M. B. ALLEN AND DANIEL I. ARNON[2]

DEPARTMENT OF PLANT NUTRITION, UNIVERSITY OF CALIFORNIA, BERKELEY 4, CALIFORNIA

Many members of the blue-green algae (Myxophyceae) possess the ability to fix molecular nitrogen. This property, combined with their photosynthetic habit of life, places these algae among the most completely autotrophic living organisms. Organisms with these properties are obviously desirable objects for investigations of photosynthesis, nitrogen fixation, and the possible relationships between these two synthetic processes. However, the blue-green algae are commonly regarded as slow-growing forms which are difficult to maintain in culture. Although there are a few reports of algae of this group which grow at a rapid rate with combined nitrogen [Emerson and Lewis (13), Kratz and Myers[3] (18)], the yields commonly reported for nitrogen-fixing blue-greens vary from 0.3 to 2.0 gm dry weight of cell material per liter of culture medium in periods of time varying from 20 to 60 days (4, 14, 24). Even in those cases where rapid growth has been reported, it has not been shown that high cell yields can be obtained in a short time. For example, the Chroococcus grown by Emerson and Lewis (13) produced 60 mg dry wt per liter of culture in 5 to 7 days. By contrast, the cultures of Chlorella and Scenedesmus commonly used in laboratory studies give yields of 5 to 10 gm dry wt per liter in 5 to 6 days (6, 7, 11). The small yield obtained with the Myxophyceae and the reputed difficulty of growing these algae has prevented them from being used in studies of photosynthetic nitrogen fixation (19).

Since dense growths of blue-green algae can appear in a short time under natural conditions, it appeared possible that many of the low yields for these organisms reported in the literature were the result of unfavorable physical or nutritional conditions for growth. The importance of favorable physical conditions for good results in the culture of Myxophyceae has previously been pointed out (1). Several unsolved problems in the nutrition of blue-green algae were also apparent. Was calcium required for growth with combined nitrogen or was it only needed for nitrogen fixation (4)? Could calcium be replaced by strontium (4; cf. 27)? Could molybdenum be replaced by vanadium (10)? Were there other inorganic nutrients required by these algae?

As a result of investigation and clarification of these points we have now found that under favorable conditions the growth of *Anabaena cylindrica* Lemm., a filamentous, nitrogen-fixing member of the Myxophyceae, is such that 7 to 8 gm dry wt of cells per liter of culture medium can be obtained in 6 days (3), an increase of approximately 200 fold over the yield previously reported for this and similar organisms (4, 14, 29).

The high yields obtained for *Anabaena cylindrica* (and for other blue-green algae not discussed here) have resulted not from the attainment of higher growth rate constants (K) than those previously reported by other investigators but rather from the maintenance of exponential growth rates at high cell densities. The purpose of this paper is to discuss the nutrient requirements and the physical conditions of the environment under which the vigorous growth of nitrogen-fixing blue-green algae was obtained.

METHODS

The alga used in these studies was Fogg's strain of *Anabaena cylindrica* Lemm. (14). It was found possible to obtain a reasonably uniform suspension of filaments by growing the culture with continuous shaking (see below). In most cases, uniform samples for inoculation of fresh cultures could be obtained by

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[3] In a paper which appeared after this manuscript was submitted for publication, these authors (Amer. Jour. Bot. 42: 282–297. 1955) report for *Anacystis nidulans* a growth constant (K) of 3.55 at cell density of ca 0.4 gm dry wt per liter of culture.

pipetting aliquots from a mature culture; 0.1 to 1.0 ml samples, depending on the density of the culture, were used for inoculation. If lumpy cultures were obtained, a uniform suspension of filaments for use as inoculum was prepared by filtering a portion of the culture through a thin mat of glass wool.

Cultures were grown in Roux bottles, on an illuminated shaker, at ca 25° C, with 5 % CO_2 in air passing over the liquid; 250 ml of medium was used in each bottle; the illuminated area was 220 cm^2. The apparatus is described elsewhere (6, 7). Unless otherwise described, the following nutrient solution was used: 0.001 M $MgSO_4$, 0.0005 M $CaCl_2$, 0.004 M NaCl, 0.002 M K_2HPO_4, and a micronutrient supplement based on the A4 and B7 solutions (9) which supplied the following final concentrations of metals: Fe [as the EDTA complex (7)] 4 ppm Mn (as $MnSO_4 \cdot 4\,H_2O$) 0.5 ppm, Mo (as MoO_3) 0.1 ppm, Zn (as $ZnSO_4 \cdot 4\,H_2O$) 0.05 ppm, Cu (as $CuSO_4 \cdot 5\,H_2O$) 0.02 ppm, B (as H_3BO_3) 0.50 ppm, V (as NH_4VO_3) 0.01 ppm, Co (as $Co(NO_3)_2 \cdot 6\,H_2O$) 0.01 ppm, Ni (as $NiSO_4 \cdot 6\,H_2O$) 0.01 ppm, Cr (as $Cr_2(SO_4)_3K_2SO_4 \cdot 24\,H_2O$) 0.01 ppm, W (as $Na_2WO_4 \cdot 2\,H_2O$) 0.01 ppm, Ti (as $TiO(C_2O_4)_x \cdot y\,H_2O$) 0.01 ppm. The micronutrient supplement included several elements not so far shown to be essential for plant growth to ensure that growth was not being limited by an unknown element. The phosphate was autoclaved separately and added to the medium after cooling, to prevent precipitation. Potassium nitrate, 0.02 M, was added for experiments in which growth with combined nitrogen was measured.

All media were prepared with glass-distilled water. For experiments on Mo and V requirements, the salts used were purified by treatment with $CaCO_3$ and CuS, as described elsewhere (7, 17). The salts used in Ca and Sr experiments were C.P. grade purified by three recrystallizations from hot water. For other experiments, C.P. salts were used without further purification. The methods of cleaning glassware and other experimental details are given elsewhere (7, 17).

The dry weight of the algal cells was used as a measure of growth. Since the medium used contained only ca 0.6 gm dry wt of salts per liter, growth was determined by drying 30 to 50 ml aliquots of the culture, evaporating to dryness, weighing, and correcting for the weight of salts in the medium. Growth figures obtained in this way are slightly low, since algae contain 5 to 10 % ash (20), but the error is small for an organism which obtains both its C and its N from the atmosphere.

For measurements of nitrogen in the cells and in the medium, the algae were removed by centrifuga-

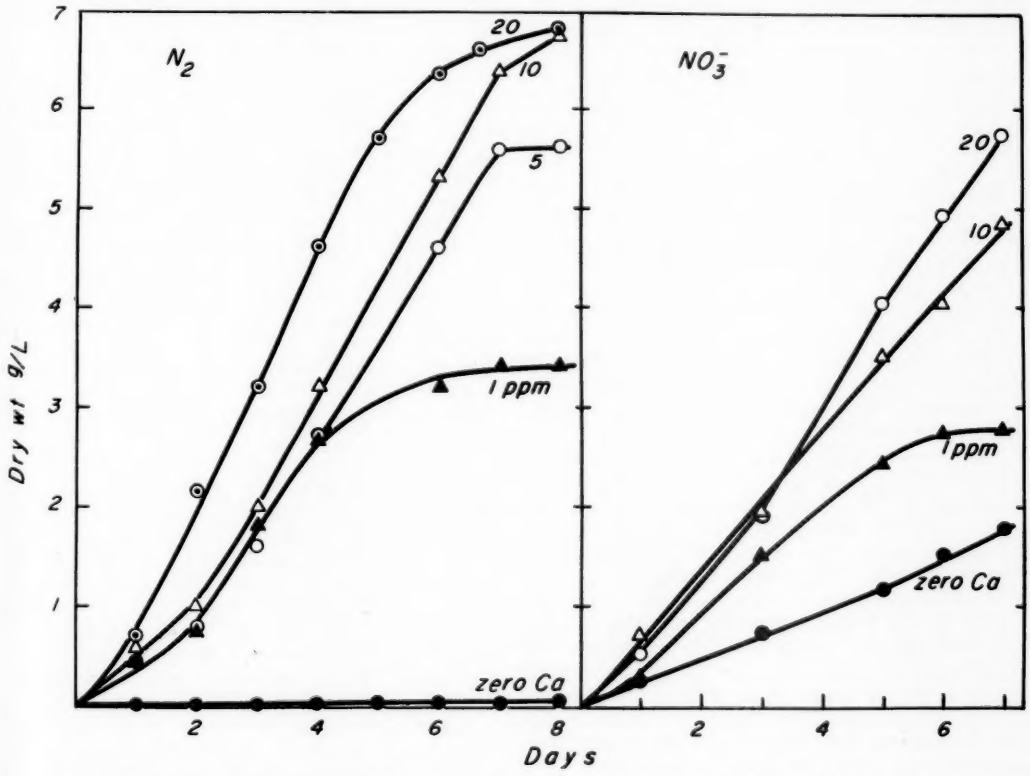

FIG. 1. The calcium requirement of *Anabaena cylindrica*. Light intensity 7500 lux. Calcium concentrations (ppm) are shown on the curves.

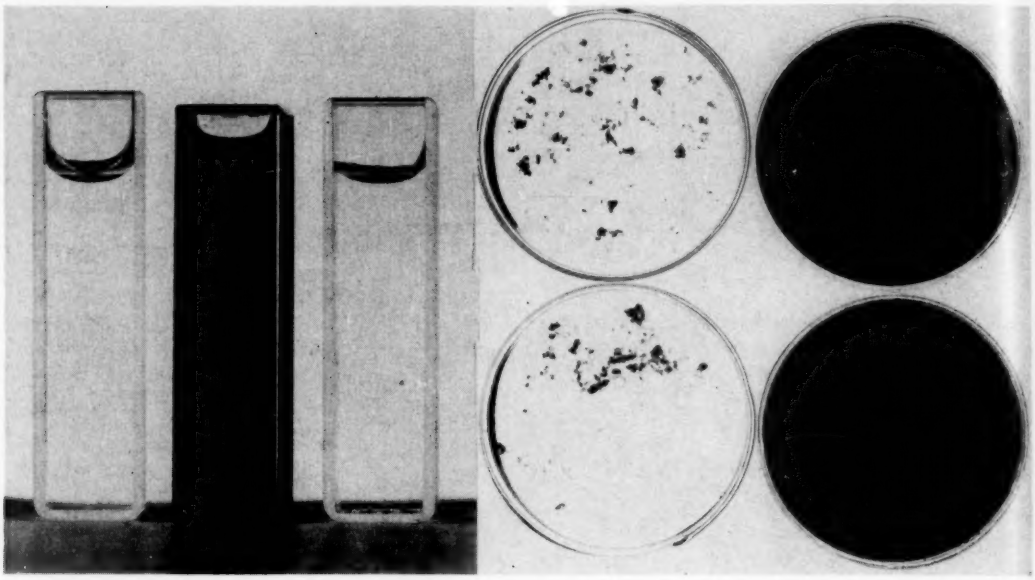

FIG. 2 (*left*). Effect of Ca and Sr on growth of Anabaena. Seven-day cultures. Light intensity 7500 lux. Left, no Ca or Sr added; center, 20 ppm Ca added; right, 20 ppm Sr added.

FIG. 3 (*right*). Effect of Mo and V on growth of Anabaena. Cultures on right received 0.01 ppm Mo and 0.01 ppm V, those on left received 0.01 ppm V. Light intensity 7500 lux.

tion and the nitrogen in the cell mass and the supernatant liquid determined by the microkjeldahl method (12).

RESULTS

NUTRITION: Little systematic work has heretofore been done on the nutritional requirements of Myxophyceae [cf. (1) for a review of the literature]. The proportions of the major nutrients used in the present study were based, in the main, on those which have been found to give satisfactory growth of higher plants (8) and of green algae (6, 7). Since these quantities of nutrients were also favorable for growth of the blue-green algae, the effects of varying the proportions of the major nutrients have not been extensively investigated here.

The importance of an adequate supply of nitrogen, molecular or combined, in obtaining good yields should, however, be emphasized. Since healthy algae contain 5 to 7 % N, calculations show that 0.02 to 0.04 M nitrate is required to support a population of 6 to 8 gm dry wt/l when the organisms are growing with combined nitrogen. When the algae are growing with molecular nitrogen, the yield obtained may be limited by the rate of diffusion of nitrogen to the cells unless provision is made by agitation of the culture, growth in thin layers of liquid, or by a combination of both measures, for free access of air to the organisms. This point will be discussed more extensively below.

Two elements, Ca and Mo, were of especial interest, since it has been reported that, although both are needed for growth of Myxophyceae, Ca could be replaced by Sr (4) and Mo by V (10). Since no other cases are known as yet in which an essential element for plant growth can be completely replaced by another (5), an examination of the requirements of Anabaena for these nutrients was made.

Calcium was shown to be required for the growth of Myxophyceae by early investigators of the physiology of these algae (23). In spite of one report that Ca is not needed (16), based on cultures in which the total growth was small, later work has generally substantiated the early results (1). However, Allison et al (4) claimed that Ca was needed by *Nostoc muscorum* only when this alga was growing with molecular nitrogen, not when nitrate was the nitrogen source.

As shown in figure 1, Ca is needed by Anabaena whether it is growing with molecular or with combined nitrogen. At least 20 ppm Ca was needed for optimum growth. Anabaena thus resembles the higher plants in requiring calcium in relatively large amounts, in contrast to the green algae, which can grow with micronutrient quantities of this element (22, 25, 27). The slight growth in the absence of added Ca with nitrate as N source may reflect the presence of a small amount of Ca contamination in the KNO_3, in spite of recrystallization of this salt.

Figure 2 shows that calcium canont be replaced by strontium for the growth of *Anabaena cylindrica*. The evidence for calcium as an irreplaceable essential element (5) for the growth of blue-green algae was obtained with purified nutrient salts under conditions

favoring vigorous growth and high cell density. Such conditions are desirable for demonstrating the nutrient requirements of plants.

Similarly, as shown in figure 3, the requirement of Anabaena for molybdenum was confirmed, and it was shown that V cannot replace Mo. Examination of the experimental results published by Bortels (10) shows that the evidence for the replaceability of Mo by V was not as definite as it has been accepted to be. Bortels studied 18 cultures of various Myxophyceae. The growth of 13 of these was stimulated by Mo, but not by V, two of them were stimulated by both Mo and V, and three did not respond to the addition of either of these elements. The two cultures which responded to both Mo and V gave yields of 0.48 and 0.28 gm dry wt/l respectively, in the absence of added Mo or V. Addition of Mo raised the yields to 0.98 and 0.74 gm/l, while addition of V resulted in 0.65 and 0.57 gm/l. In the present study, growth of Anabaena without Mo or V resulted in a yield of 0.28 gm/l, addition of V resulted in a yield of 0.36 gm/l, and addition of Mo raised the yield to 5.8 gm/l.

Supply of Nitrogen and Carbon Dioxide: Since Anabaena growing with molecular nitrogen obtains both its C and its N from the gas phase above the culture, it is clearly necessary to provide for free access to these gases to the cells. In our experiments this was accomplished by passing a mixture of 5 % CO_2 in air over the liquid surface and shaking the cultures to ensure mixing of the gas with the liquid. Aeration was further favored by growing the cultures in thin layers of nutrient medium in Roux bottles, which provide a large liquid-gas interface. Under these conditions, growth with molecular nitrogen was as fast as growth with nitrate, as shown in figure 4.

Shaking, while desirable for obtaining uniform cultures, is not strictly necessary for good growth when a large liquid-gas interface is available. For example, in one experiment a shaken culture yielded 7.1 gm/l of dry Anabaena cells, whereas a parallel culture, treated in the same manner except that it was not shaken, gave a yield of 6.9 gm dry wt/l. The shaken culture, however, was uniformly blue-green, whereas the unshaken one, which had grown only at the gas-liquid interface, was partly old and bleached out while other portions were still growing. Thus it appears desirable to shake cultures which are to be used for experimental purposes in the laboratory, but provision of a large surface exposed to air, without agitation, might be satisfactory if it were desired to establish mass cultures of nitrogen-fixing blue-green algae. It seems likely that the improvement of growth of filamentous blue-green algae previously noted on introduction of a solid substratum such as glass wool into the culture medium (1) resulted from the more disperse growth of the algae under these conditions, with consequent better access of gases to the cells.

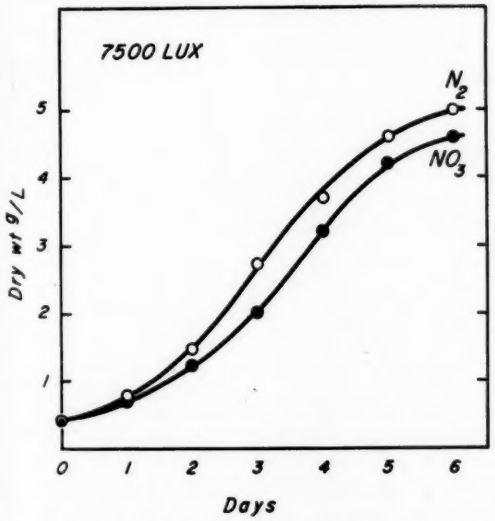

Fig. 4. Growth of Anabaena with molecular and combined nitrogen.

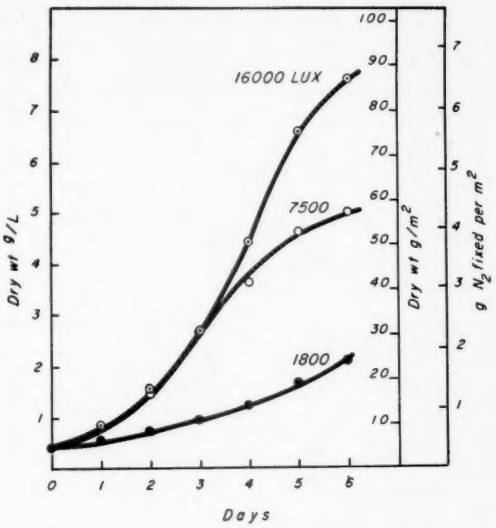

Fig. 5. Effect of light intensity on growth of Anabaena. N_2 as nitrogen source.

Intensity and Duration of Light: It has been common practice in the past to grow blue-green algae at rather low light intensities, from 300 to 3000 lux. Some workers have claimed that these algae are injured by high light intensities (4). Our experiments have shown, however, that under good conditions of mineral nutrition and with an adequate supply of nitrogen and CO_2 Anabaena can grow well at high light intensity, and that the yield of cells increases with increasing light intensity up to at least 16,000 lux, as illustrated in figure 5.

The question whether algae, under controlled laboratory conditions, grow better when given a dark period each day, or whether maximum growth per unit time is obtained when the cells are continuously illuminated has never been fully resolved. Earlier

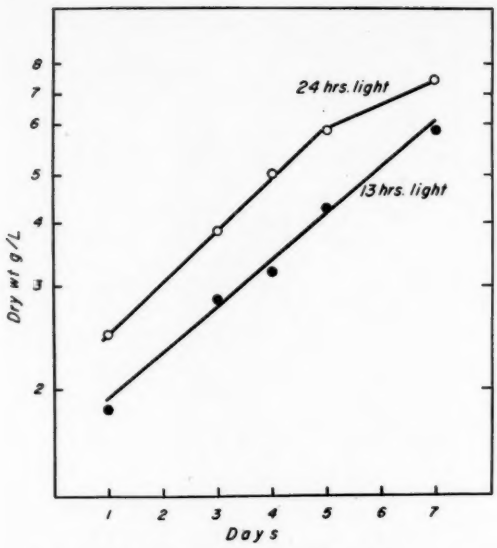

FIG. 6. Comparison of growth of Anabaena in continuous and intermittent light. Light intensity 16,000 lux. N_2 as nitrogen source. The upper curve shows growth in continuous light, the lower, growth with 13 hrs light and 11 hrs darkness each day. The ordinate is a logarithmic scale.

workers tended to give their cultures a dark period; Allison et al (4), for example, reported slightly better growth of *Nostoc muscorum* with 18 than with 24 hours of light each day. Recent practice, however, has been to grow cultures of algae under continuous illumination (21). Our experiments with Anabaena have shown that the rate of growth, under the conditions tested, was essentially the same in cultures receiving 11 hrs of darkness and 13 hrs of light as in those receiving 24 hrs of light each day, as is illustrated in figure 6. The cultures in continuous light had a shorter lag period, but once active growth had started there was no effect of the dark period on growth. Similar results have been previously obtained for Chlorella (2), suggesting that this behavior may be found in many algae. Similar results have been obtained by other workers; Tamiya et al (26) observed no retardation of growth of *Chlorella ellipsoidea* on interposition of a 12-hour dark period each day as long as the cells were growing logarithmically. It follows from these results that the yields of algae which might be expected under natural conditions with alternating day and night periods need not be very different from those obtained in continuous light in the laboratory.

DISCUSSION

It is evident from the growth figures reported in this paper that there is no longer any need to consider the common nitrogen-fixing Myxophyceae as slow-growing algae. Further emphasis of this point is provided by figure 7, where the growth of *Anabaena cylindrica* is compared with that of the green alga Scenedesmus (strain D_3). Although the growth of Scenedesmus shown here is higher than most figures previously published, the yield of Anabaena, while somewhat lower, is comparable to that of the green alga. The maximum daily increment of Anabaena growth was 2.0 gm/l, or 26 gm dry wt/m^2 of illuminated surface. The corresponding figures for Scenedesmus were 2.8 gm/l, or 36 gm/m^2.

These high yields have not been obtained by any increase in the growth rate constant of the alga, but rather by the maintenance of high rates of growth in dense cultures. The growth rate constant is an expression of the potentialities of an organism. Suitable conditions for the realization of these potentialities must be provided if high yields are to be obtained. Thus Fogg (14) reported a growth rate constant of 0.70 to 0.75 for this strain of *Anabaena cylindrica*, which agrees well with values which we have obtained, but his cultures required 60 days to reach a density of 1.7 gm dry wt/l. Fogg obtained exponential growth of the alga only in cultures so thin that the only measure of growth which could be used was microscopic observation of increases in filament length. In our cultures, on the other hand, exponential growth continued up to approximately 6 gm dry wt/l (fig 6). The growth rate constant of an

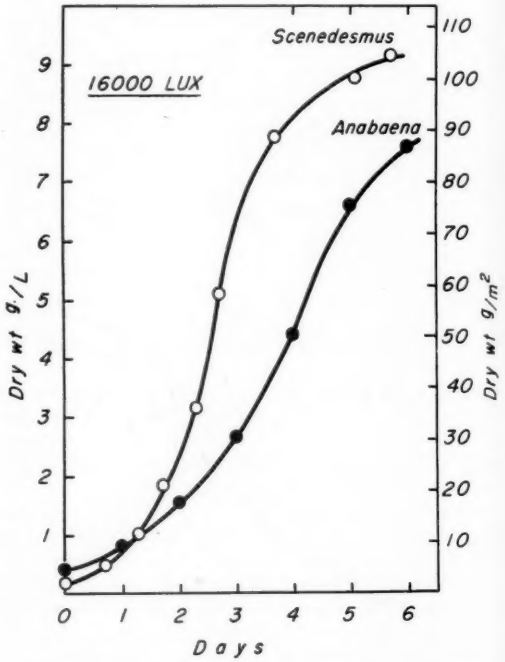

FIG. 7. Comparison of growth of Scenedesmus and Anabaena. Medium and experimental conditions for Anabaena as described in Methods; Scenedesmus was grown as described elsewhere (6, 7).

alga may be of less importance for obtaining high cell yields than the provision of physical and nutritional conditions which permit exponential growth at high cell densities.

Several workers have shown that blue-green algae excrete a portion of the nitrogen which they fix into the medium in the form of amino acids and peptides (15, 28). According to Fogg (15), as much as 30 to 40 % of the nitrogen fixed by *Anabaena cylindrica* appears in the medium in soluble form. Under our conditions, however, most of the nitrogen remained in the cells, no more than 7 to 8 % appearing in the medium. Fogg concluded (15) that the excretion of nitrogenous substances by Myxophyceae is favored by deficiencies of micronutrient elements, especially iron. The influence of nutrition and other conditions of growth on the excretion of nitrogen compounds by Anabaena is being studied further.

Summary

The capacity for growth of blue-green algae with molecular nitrogen has been found to be of the same order of magnitude as the growth of vigorously growing green algae with nitrate nitrogen.

An investigation of the nutrient requirements of the blue-green alga *Anabaena cylindrica* Lemm. disclosed that calcium was essential for growth whether nitrate or molecular nitrogen was used. Previous reports had suggested that calcium was required with molecular nitrogen only.

The specificity of two elements previously reported to be replaceable in the growth of blue-green algae was established. Calcium was not replaceable by strontium nor molybdenum by vanadium.

Growth of Anabaena increased with increasing light intensity up to 16,000 lux. An intensity of 16,000 lux for 13 hrs each day permitted as rapid growth as 24 hrs of illumination.

Under the conditions described, a yield of Anabaena of 7 to 8 gm dry wt/l of culture solution in 5 to 6 days was obtained with either nitrate or molecular nitrogen.

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CONVERSION OF CARBON-14-LABELED UREA INTO AMINO ACIDS IN LEAVES[1,2]

GEORGE C. WEBSTER, J. E. VARNER[3] AND A. N. GANSA
KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

It has become increasingly evident that the foliar application of urea to many different plants represents an extremely valuable nutritive technique. The urea is absorbed, as such, by the leaf cells and is then presumably hydrolyzed by cellular urease to carbon dioxide and ammonia. Unfortunately, our knowledge of urea metabolism in plants, based on actual experimental evidence, is very small. Especially lacking is information on the effect of urea nutrition on cellular nitrogen metabolism. In this connection it should be pointed out that evidence for the universal occurrence of even the presumed first step in the metabolism of urea, its hydrolysis to carbon dioxide and ammonia, is not at all clear. For example, Walker (6) has reported that Chlorella cells grown with urea as the sole nitrogen source contain no detectable urease. In spite of this they assimilate urea readily and grow, with a concurrent increase in their levels of cellular arginine and argino-succinic acid. Likewise, Williams and Sharma (8) have reported that urease essentially disappears from seedlings of *Citrullus vulgaris* within nine days of germination.

In contrast, Hinsvark et al (2) have concluded from isotopic studies that the leaves of a number of higher plants hydrolyze urea readily. It seems, therefore, that further knowledge of the metabolic fate of urea in plant cells is needed. This problem has recently been investigated by Boynton et al (1) who have reported that treatment of apple leaves with urea results in increased levels of total, amino, and amide nitrogen. The present communication reports the results of a study carried out on the metabolism of urea-C^{14} in leaves of bean plants.

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[2] Supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Company.
[3] Present address: Department of Agricultural Biochemistry, The Ohio State University, Columbus, Ohio.

EXPERIMENTAL MATERIAL AND METHODS

The experimental material (*Phaseolus vulgaris*) was grown in the manner described by Rogers (5). Thirty grams of young leaves were detached from the plants and mechanically agitated under weak (50 fc) white light at 23° C for six hours while completely submerged in 200 ml of 0.01 M urea-C^{14} having a total activity of 1 mc. In a parallel experiment, 30 gm of young leaves were incubated with 0.01 M $NaHC^{14}O_3$ with a total activity of 1 mc in a solution containing 0.01 M NH_4Cl. The incubation was stopped by quickly freezing the leaves in a mixture of solid carbon dioxide and acetone. Out of a total of 2,220,000,000 cts/min in each incubation solution, the leaves absorbed a total of 15,300,000 cts/min from the urea-C^{14} solution and 28,500,000 cts/min from the $NaHC^{14}O_3$ solution. The leaves were ground in a Waring blendor with 100 ml of water, filtered, and the heavy cellular particles sedimented at 18,000 × g. The remaining clear supernatant was treated with two volumes of acetone to precipitate protein. The protein was sedimented by centrifugation in a Servall refrigerated centrifuge. The protein-free supernatant was reduced to a small volume in a low temperature rotating evaporator. The protein obtained by acetone treatment was combined with that of the sedimented cellular particles and the total protein hydrolyzed in a sealed tube at 150° C with 20 % HCl. To the amino acids obtained from both the cellular extracts and the protein hydrolysates, a mixture of 17 carrier amino acids was added. The amino acids were separated either on a Dowex-50 column or on a combination of ion exchange columns by the method of Hirs, Moore and Stein (3) with the aid of an automatic fraction collector (Autonomos Instruments, Columbus, Ohio). The two ml fractions were evaporated to dryness on copper planchets, and the radioactivity of each determined with a Nuclear-Chicago G-M tube

and a Model 183 scaling unit equipped with an automatic sample changer. The amino acids eluted from the column were identified not only by their appearance at the elution points given by Hirs, Moore and Stein (3), but also by comparison of the paper chromatographic behavior of each extracted amino acid with the behavior of authentic samples of amino acids. Amino acids were quantitatively determined with ninhydrin after chromatographic separation as described previously (8). This technique serves also to determine glutamine and asparagine.

Results

The incorporation of the carbon of $NaHC^{14}O_3$ and and of urea-C^{14} respectively into the free amino acids of bean leaves after 6 hours is presented in table I. In general agreement with the results of Racusen and Aronoff (4), the greatest amounts of radioactivity in the free amino acids are found in aspartate, glutamate, asparagine, glutamine, serine, glycine and alanine. Only negligible radioactivity is present in the other free amino acids in the leaves. It is of interest to note that the total activity incorporated into each of the free amino acids is greater in the case of incubation with urea-C^{14} than with $NaHC^{14}O_3$ plus NH_4Cl. This occurs despite the fact that more radioactivity from $NaHC^{14}O_3$ is taken up by the cells than from urea-C^{14}. The radioactivity of $NaHC^{14}O_3$ appears to a much greater extent in sugars and starch, however.

The incorporation of the carbon-14 of the two assimilated substances, urea and bicarbonate, into the protein-bound amino acids is also presented in table I. Although the same general pattern of radioactivity in aspartate, glutamate, glycine, serine and alanine is found here, there is also radioactivity in a number of other amino acids. This suggests that these amino acids are utilized for protein synthesis as soon as they are formed.

It should be noted that in both the free and protein-bound amino acids, the general pattern of activity is the same whether the radioactive carbon is assimilated from $NaHCO_3$ or from urea. This is consistent, in agreement with the conclusion of Hinsvark et al (2), with the idea that the first step in urea metabolism in these leaves may be its hydrolysis to CO_2 and NH_3.

There are, however, several differences in the response of the bean leaves to the carbon of CO_2 and of urea. While a fairly large amount of free urea accumulates in the tissue during incubation with urea-C^{14} (table I), no detectable radioactive urea is formed during the metabolism of $C^{14}O_2$ and NH_3 by the leaves. If the ornithine cycle operates in plants as Racusen and Aronoff (4) have suggested, then the urea produced from the action of the cycle does not accumulate to detectable levels. Alternatively, the reactions of the cycle may occur only in part and without actual cycling (for example, as a system for the synthesis of arginine).

In agreement with the radioactivity measurements given in table I, urea causes higher levels of free glutamate, aspartate, serine, glycine and alanine than does a mixture of $NaHCO_3$ and NH_4Cl (table II). That this increase is not due to an enhanced breakdown of protein is indicated by the finding that the level of protein in leaves treated with urea is also slightly higher than in the leaves given $NaHCO_3$ and NH_4Cl. In contrast, the levels of glutamine and asparagine are markedly lower after urea treatment than after treatment with $NaHCO_3$ and NH_4Cl.

Discussion

The most important result of this investigation is the demonstration that the pattern of incorporation of the carbon of urea is essentially the same as that of the carbon of $NaHCO_3$. This suggests that urea may be metabolized in bean leaves by its hydrolysis to CO_2 and NH_3.

Table I

Incorporation of C^{14} of Urea and $NaHCO_3$ into the Free and Protein-bound Amino Acids of Bean Leaves

Compound	Total radioactivity (cts/min)			
	Free amino acids		Protein-bound amino acids	
	Urea	$NaHCO_3$	Urea	$NaHCO_3$
Alanine	61,000	41,000	23,000	15,000
Arginine	.....	.....	5,000	3,000
Aspartate	350,000	179,000	187,000	167,000
Asparagine	150,000	201,000	.....	.....
Cysteine	.....	.....	1,400	1,300
Glutamate	250,000	147,000	199,000	151,000
Glutamine	280,000	290,000	.....	.....
Glycine	368,000	200,000	51,000	56,000
Histidine	.....	.....	550	480
Leucine	.....	.....	600	450
Lysine	.....	.....	500	200
Methionine	.....	.....	1,600	350
Phenylalanine	.....	.....	600	200
Proline	.....	.....	100	80
Serine	99,000	57,000	37,000	20,000
Valine	.....	.....	3,500	3,000
All other amino acids	33,000	15,000	.....	.....
Urea	1,596,000	.....	.....	.....

Table II

Free Amino Acid and Amide Contents of Bean Leaves after Incubation with Urea and with $NaHCO_3 + NH_4Cl$

Amino acid or amide	Total amino acid content (μM/gm tissue)	
	Urea	$NaHCO_3 + NH_4Cl$
Alanine	0.51	0.43
Aspartate	1.35	0.58
Glutamate	1.99	1.03
Glycine	0.38	0.25
Serine	0.89	0.61
Glutamine	0.83	1.38
Asparagine	0.56	1.55

The quantitative differences in incorporation from the two substrates into amino acids, protein and amides would appear to be a reflection of the fact that urea is obviously (table I) hydrolyzed more slowly than it is absorbed, with the result that the CO_2 and NH_3 made available to the plant by urea hydrolysis are released at a slow but steady rate. In contrast, the mixture of $NaHC^{14}O_3$ and NH_4Cl gives a large increase in ammonia concentration as soon as it is absorbed. It is well established that such an increase (above that which the cell can use for amino acid synthesis) is generally conjugated by the cell in the form of glutamine and asparagine. The differences in the levels of total amino acids and amides observed here would appear to be most probably the result of the differences in ammonia levels in the cells as a result of rate limitation by urease activity.

Summary

The incorporation of the carbon of urea-C^{14} into amino acids has been demonstrated in bean leaves. The pattern of incorporation is almost identical to that obtained with $NaHC^{14}O_3$. Incubation of bean leaves with urea for a six-hour period results in a higher amino acid and protein content than is obtained with a mixture of $NaHCO_3$ and NH_4Cl. Higher levels of glutamine and asparagine are obtained with $NaHCO_3 + NH_4Cl$, however.

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BIOASSAY OF MERCURY VAPOR ARISING FROM A PHENYL MERCURY COMPOUND[1]

A. E. DIMOND and PAUL E. WAGGONER

Department of Plant Pathology and Botany, The Connecticut Agricultural Experiment Station, New Haven, Connecticut

Mercury vapor has been detected quantitatively by a bioassay in connection with a study on the liberation of mercury vapor from paint films containing phenyl mercury compounds. This assay depends upon the ability of small quantities of mercury vapor to prevent foliar abscission of bean plants in vitro. Although chemical methods exist for detecting mercury vapor, these methods were found unsuitable in the present problem. Thus palladium chloride paper (3) darkened when confined with paint films containing no mercury or with portions of living plants. Selenium sulfide paper (3) was likewise not sufficiently selective and was less sensitive than palladium chloride paper to mercury vapor. Detection of mercury vapor through its absorption of ultraviolet radiation of wavelength 2537 Å. is the basis of a vapor detector manufactured by the General Electric Co. Although this instrument is sensitive, it is not generally available in laboratories. The bean assay is presented here because it requires no special equipment, is simple, rapid and inexpensive, is quantitative in the range from 0.06 to 0.9 mg mercury per m^3 of air, and is selective for mercury vapor in the presence of materials which interfere with other standard spot tests for mercury referred to above.

[1] Received April 18, 1955.

Materials and Methods

The assay is patterned after that developed by Addicott et al (1) and involves the use of explants from trifoliate leaves of Pinto beans. The explants are of two types: (a) the pulvinus and 10 mm of the petiolet of the central leaflet and (b) the 2 pulvini of the lateral leaflets and the attached 10 mm of the petiole of the trifoliate leaf. The two types of explant respond in the same manner to mercury vapor and are used in pairs in the assay. Thus each trifoliate leaf provides material for three possible abscissions. The stalks of the explants are placed directly on 3 % water agar in a Petri dish in which a central channel has been cut out and over which the pulvini are placed. A source of mercury vapor is placed in the dish out of contact with the explants and the Petri dish is sealed with a wide rubber band to prevent loss of mercury vapor. After incubation in daylight for 3 or 4 days at room temperature (ca 24° C), the terminal portion of the control explants

have become a pale yellow translucent color and will fall off if touched lightly. If presoaked in solutions of indole acetic acid (IAA) or 8-quinolinol or exposed to either 8-quinolinol or mercury vapor, the explants either fail to abscind or abscission is delayed. However, mercury vapor causes a general softening of the petiolar tissue after 3 or 4 days whereas 8-quinolinol or IAA cause the tissue to remain green and fully turgid. By this qualitative difference in response, other materials which delay abscission can be discriminated from mercury vapor.

Results and Discussion

Response to Metallic Mercury Vapor: An experiment tested the difference in response of bean explants to mercury vapor and laboratory air. Three pairs of test objects were placed in each of four dishes, two per treatment. Small droplets of mercury were placed on the moist agar surface, out of contact with the explants in two of these dishes and nothing was added to the others. Of a total of 18 possible abscissions per treatment, none had occurred after seven days' exposure to mercury vapor, whereas in the controls there were none after one day, 8 abscissions after 2.5 days, 15 after 3 days' and 18 at the end of 4 days' incubation. Thus abscission was complete in control after 3.5 to 4 days. Explants exposed to mercury vapor became soft and slimy after the fourth day and had disintegrated without abscinding by the end of the experiment.

Response to Mercury Vapor in the Presence of IAA: Because IAA also delays abscission in the bean assay (4), an experiment was made to compare the effects of no hormone, and presoaking in 2×10^{-4} or 10^{-3} M IAA in the presence and in the absence of mercury vapor. Three pairs of explants in duplicate for each of the 6 possible treatments were employed, making a total of 18 possible abscissions per treatment. In the absence of IAA, results were the same as reported in the paragraph above. In the presence of mercury vapor there was no abscission within 7 days, whether or not IAA was present. The tissues exposed to mercury vapor became soft and slimy whether or not IAA was present: the response was characteristic of mercury injury and not of IAA. In the absence of mercury, IAA at 2×10^{-4} M delayed but did not prevent abscission, there being 8 of 18 possible at the end of 4 days and 15 abscissions at the end of seven days. Similarly at 10^{-3} M IAA there was no abscission at the end of four days, one at the end of 6 days and no further change at the end of 7 days. Evidently, if IAA is present in the mercury assay, it does not interfere because the response is that of material exposed to mercury vapor alone. However, IAA delays the abscission of explants when mercury vapor is not present.

Response to Paint Films: To determine the effect of phenyl mercury compounds in paints on the bean response, a sample of white paint was employed. To one portion of this paint, the manufacturer had added di(phenyl mercuric) dodecenyl succinate (DPMDS), so that the paint contained 0.24 % mercury on a metallic basis. The second portion of the paint contained no mercury. Each of these paints was applied with a brush to 9-cm circles of filter paper and the film was allowed to dry 4 days. Then the painted surfaces or unpainted filter papers were confined in Petri dishes during a 4-day bean assay. In this time there was no abscission by bean explants confined with the mercury-containing paint, whereas abscission was complete for control bean explants and for those confined with the paint containing no mercury. Evidently a volatile material was liberated from the mercury-containing paint which prevented the abscission of the bean explants.

Evidence that the Volatile Material Is Mercury Vapor: To determine the nature of the volatile material affecting the bean assay which arose from the paint with a phenyl mercury additive, filter paper circles were coated with enamel grinding oil, DPMDS, the white paint containing DPMDS, a mixture of DPMDS and enamel grinding oil or were left uncoated. After drying for 4 days, these filter paper circles were rolled up and placed in the bottom of screw top test tubes. Metallic mercury was also placed in a pool in other tubes.

Metallic mercury vapor was detected in several ways. After 24 hours the atmosphere in one series of tubes was analyzed with a General Electric vapor detector. Two other series of tubes contained palladium chloride paper or selenium sulfide paper, both held by the screw top and therefore not in contact with the mercury or mercury coating. After 48 hours the response of the palladium chloride and the selenium sulfide papers was noted and these responses ranked in increasing order of darkening. A fourth method, specific for mercury, involved trapping mercury vapor as an amalgam in gold foil which was confined in tubes with the source of vapor but out of direct contact with the source. After 3 days' incubation the gold foil was removed and analyzed for mercury content by the method of Zimmerman and Crocker (5). The gold foil was placed in a small glass tube, the tube drawn at the upper end to a capil-

Table I

Liberation of Mercury Vapor from Metallic Mercury and Mercury Compounds Material

	SeS paper*	$PdCl_2$ paper*	Gold foil method*	Vapor detector mg Hg/m³ air
Check	0	0	0	0
Enamel grinding oil	1	2	0	0
DPMDS	2	1	1	0.66
Mercury-containing paint	3	3	2	1.56
DPMDS plus enamel grinding oil	5	4	3.5	2.42
Metallic mercury	4	5	3.5	2.30

* Responses ranked in increasing order of intensity, no response being indicated as 0.

lary and allowed to cool. Then the gold foil in the bottom was heated to vaporize any mercury amalgamated by the foil. The bottom of the tube containing the gold foil was then broken, and the foil removed. The upper portion containing the capillary and any mercury condensed inside it was sealed in a screw top tube containing iodine crystals. A deposit of red mercuric oxide in the neck of the capillary indicated that metallic mercury vapor had been trapped by the gold foil.

Results of all these examinations indicate that mercury vapor is liberated when the materials containing mercury were present (table I). The vapor detector is a reliable measure of mercury vapor in the absence of interfering solvent vapor (2). The vapor detector showed no reading for paper circles coated with paint containing no phenyl mercury compound and confined in a tube for 24 hours before sampling with the detector. Responses of selenium sulfide and palladium chloride papers were only approximately related to the amount of mercury vapor as indicated by the vapor detector. The bean assay reacted to the mercury-containing materials but not to paint alone and is reliable.

Calibration of the Bean Assay for Mercury Vapor: To determine the response of the bean explants to varying concentrations of mercury vapor, sheets of filter paper were painted with the mercury-containing paint and allowed to dry for 4 days. Rectangles varying in size from one to 64 cm^2 were cut out, 7 replicates for each area and 6 different areas. The 42 painted surfaces were confined in separate screw top tubes for 24 hours, and the amount of mercury vapor in the tubes determined with a previously calibrated General Electric vapor detector. The individual pieces of painted paper were then used in Petri dishes of comparable volume to the screw top tubes during a 4-day bean assay. In this assay 8 pairs of explants were used in each dish, permitting 24 possible abscissions per dish. Following the assay, the painted surfaces were again confined in clean screw top tubes for 24 hours and the amount of mercury vapor again analyzed with the calibrated vapor detector. Thus the concentration of mercury vapor in the Petri dishes during the bean assay was estimated with a vapor detector before and after the assay itself. The mean of the 7 replicate determinations of concentration of mercury vapor for each area did not vary significantly from one another when made before and after the bean assay. These determinations served as a basis for a calibration curve for the assay (fig 1). Evidently the bean assay is sensitive to mercury vapor in the range from 0.06 to 0.9 mg mercury vapor per m^3 of air.

This assay has proven useful in evaluating treatments to prevent the liberation of mercury from paint films containing phenyl mercury compounds, a matter to be reported elsewhere. The assay may prove useful in other studies concerning the effect of mercury vapor on plant tissue.

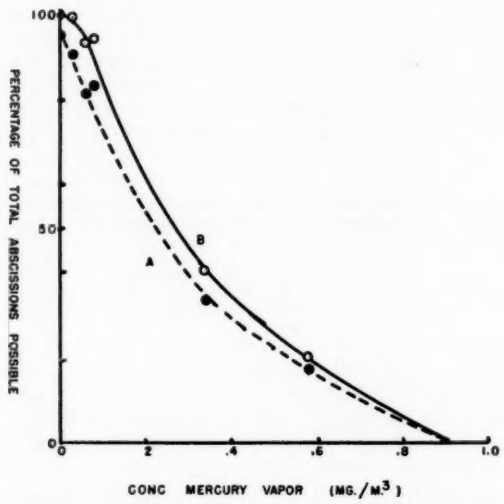

Fig. 1. Calibration curve for the bean assay in terms of mg per m^3 of mercury vapor in the atmosphere. Curve A is for a 3-day and curve B for a 4-day assay.

Summary

The assay for mercury vapor described depends upon the prevention of abscission in bean explants. This assay can be completed in four days, requires no special materials, is reasonably selective for mercury vapor, and is quantitative in the range from 0.06 to 0.9 mg mercury vapor per m^3 of air. The assay has proven useful in demonstrating the liberation of metallic mercury vapor from paint films to which phenyl mercury compounds have been added as mildew proofing agents.

Miss Barbara Wooding contributed valuable technical assistance in these studies. The authors are also indebted to Mr. Allan L. Coleman, Bureau of Industrial Hygiene, State Department of Health, Hartford, Conn., who made available for these studies a G. E. Vapor Detector which had been accurately calibrated for mercury vapor.

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INCORPORATION OF RADIOACTIVE ACETATE AND SUCROSE INTO AMINO ACIDS AND PROTEIN OF EXCISED ORGANS OF RED KIDNEY BEAN [1,2]

BRUCE J. ROGERS [3]

KERCKHOFF LABORATORIES OF BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Little information is available on rates of incorporation of C^{14}-labeled amino acids into plant protein. The first reported work was that of Arreguin, Bonner and Wood (1) in 1951 in which C^{14}-carboxyl-labeled acetate was supplied to guayule plants. The bulk of the radioactivity in the protein fraction was located in leucine, glutamate and aspartate. Boroughs and Bonner (4) incubated C^{14}-carboxyl-labeled leucine and glycine with corn and oat coleoptiles and recovered radioactive protein. More recently Webster (13) has carried out experiments on the incorporation of C^{14}-labeled glycine, glutamate, and aspartate into bean hypocotyl mitochondria. This work has been extended to show incorporation of glutamate into the proteins of several different plants (14). Racusen and Aronoff (11) supplied $C^{14}O_2$ to discs cut from soybean leaves and found considerable amounts of radioactivity in protein. In the present work the rates of incorporation of C^{14}-labeled amino acids into the protein of detached leaves, stems and roots of red kidney bean plants were studied. This is of interest for two reasons: 1) the relative rates of incorporation of amino acids into the protein of the three major organs of a particular plant have heretofore not been compared, and 2) it was desired to investigate further the factors connected with the rapid decrease in protein contents of leaves after excision (6).

In connection with the latter point, the incorporation of C^{14} into the protein-bound amino acids of the organs was studied. Both Paech (10) and Chibnall (6) have suggested that a generalized mass action relation may control the protein content of detached leaves, protein synthesis being dependent on the maintenance of a sufficiently high concentration of amino acids. Chibnall has suggested that the formation of alpha-keto derivatives of the amino acids may disturb this equilibrium. The results of Wood and Cruickshank (15) and of Kemble and Macpherson (7) tend to support Chibnall's suggestion. It was demonstrated by these workers that the concentrations of certain amino acids decrease in detached leaves even though these amino acids are being liberated by protein hydrolysis and the total amino acid nitrogen is increasing. It is implied that the mass action relation between protein and amino acids may be deranged by the high rate of catabolism of these amino acids (15). The data led Bonner to suggest that perhaps leaves may be unable to synthesize all of the required amino acids. Other organs—probably the roots—do synthesize all of the amino acids and supply them to the leaves in the intact plant (3). This study was designed to test the possibility that the synthesis of certain amino acids is localized in organs other than the leaves.

MATERIALS AND METHODS

Red kidney bean (*Phaseolus vulgaris*) plants were grown to a height of 30 to 45 cm in the Earhart Plant Research Laboratory at a day temperature of 23° C and a night temperature of 17° C. The plants were harvested and divided into still-expanding leaves and stems and roots. Twenty-gram portions of each organ were floated partially submerged in a solution containing either C^{14}-uniformly-labeled sucrose (initial activity 67,000 cpm/ml; final activity 30,000 cpm/ml) or C^{14}-carboxyl-labeled sodium acetate (initial activity 115,000 cpm/ml; final activity 70,000 cpm/ml) for different periods of time up to 12 hours. The specific activity of the labeled sucrose was 0.17 mc/millimole, and that of the labeled acetate was 1 mc/millimole. The experiment was carried out at room temperature at light intensities of about 10 fc. At the end of each incubation period the tissues were removed from the solution, rinsed two times in water, and quick-frozen in a mixture of solid CO_2 and acetone. Each portion of frozen material was homogenized in water in a Waring blendor and the homogenate filtered. The protein in the filtrate was precipitated by addition of trichloroacetic acid (TCA)

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN AMINO ACIDS OF RED KIDNEY BEAN AFTER 12 HRS INCUBATION OF EXCISED ORGANS WITH ACETATE-1-C^{14} AND SUCROSE-C^{14}

AMINO ACID	ACETATE-1-C^{14}		SUCROSE-C^{14}	
	LEAVES	ROOTS	LEAVES	ROOTS
	cpm × 10^{-3}/millimole of amino acid			
Alanine	320	30	100	1
α-Aminobutyric acid	1	1	...	..
Arginine	10	5	6	10
Aspartic acid	100	50	150	80
Cystine	1	1	1	10
Glutamic acid	100	100	400	60
Glycine	650	250	60	50
Leucine-Isoleucine	1	4	10	4
Lysine	3	4	10	2
Methionine	1	2	3	10
Phenylalanine	1	1	20	10
Serine	40	100	150	30
Threonine	110	100	100	3
Valine	10	100	10	10

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[2] Report of work supported in part by the Polychemicals Department, E. I. du Pont de Nemours and Company.

[3] Present address: Department of Botany and Plant Pathology, Purdue University, Lafayette, Indiana.

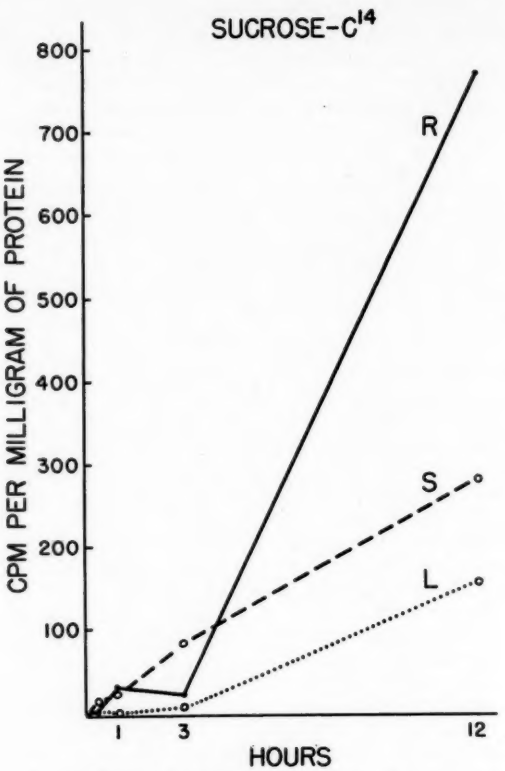

FIG. 1. The rate of incorporation of C^{14} of sucrose-C^{14} into leaf, stem and root protein of red kidney bean. R—roots; L—leaves; S—stems.

to a final concentration of 2 to 3 % and removed by low-speed centrifugation. The protein was washed in TCA, dissolved in alkali and re-precipitated by addition of TCA. This treatment was found adequate for the removal of adsorbed radioactive material. The protein was spread evenly on aluminum planchets and the incorporated radioactivity measured with a thin-window Geiger-Müller tube by standard techniques. A sufficient number of counts were taken to insure a standard error of not more than ± 5 %. Correction was made for self-absorption according to the recommendations of Calvin et al (5).

The supernatant solution was reduced in volume, placed on a Dowex-50 ion exchange column in the acid phase, and eluted by successive applications of water and HCl. The treatment sufficed to separate the free amino acids from sugars and organic acids almost entirely. The aliquots collected from the acid elution were combined and the amino acids were separated into groups by paper chromatography (8). The individual amino acids were isolated from the groups by a second series of paper chromatographs (2). This double system of chromatography served both to isolate the amino acids and further to separate them from contaminants. Each amino acid was eluted from the paper and divided into two equal aliquots. One portion was placed in a copper planchet for assay of radioactivity by means of a gas-flow counter to a probable error of ± 8 %. The amino acid concentration of the second portion was determined by the ninhydrin method of Moore and Stein (9).

RESULTS AND DISCUSSION

The results for the amino acids were calculated as cpm/millimole of amino acid and are given in table I for the 12-hour incubation period. Data are not available from the present work for all of the amino acids which are found in plants, but it is apparent that most of those found in plant protein which are measured here are synthesized by leaves. It does not seem probable, therefore, that protein loss in excised leaves can be attributed to a lack of amino acid synthesis in leaves.

The data obtained for the incorporation of amino acids into protein are shown in figures 1 and 2. A comparison may be made of the relative rates of incorporation of C^{14} into the protein of the 3 organs. After a lag period of some 3 hours large differences in the rate of incorporation of C^{14} into protein become apparent. In both the acetate and the sucrose experiments, the protein radioactivity rises much more rapidly in the root tissue than in either the stem

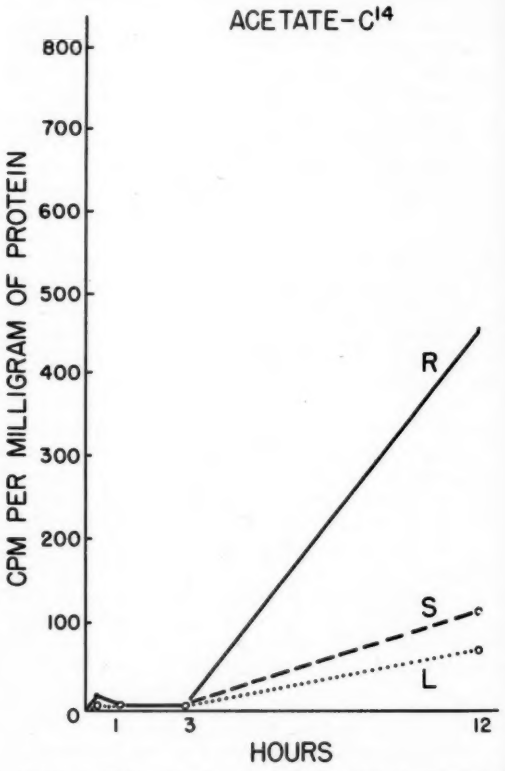

FIG. 2. The rate of incorporation of C^{14} of acetate-C^{14} into leaf, stem and root protein of red kidney bean. R—roots; L—leaves; S—Stems.

or the leaf tissue. The possibility that this might be due to higher specific activities for the amino acids of the root is ruled out from the studies on the amino acids.

The differences in rates of incorporation cannot definitely be attributed to excision on the basis of the present work. However, a comparison may be made with data on incorporation into the tissues of intact plants from the report of Vickery et al (12) on the incorporation of N^{15} ammonia into the protein of intact tobacco plants. Results obtained in the latter work show an incorporation of N^{15} into root protein which is twice that found for the incorporation of N^{15} into leaf protein. The isotope was applied to the roots, so a translocation factor may be involved. In the work reported on here, the ratio of incorporation into root protein to incorporation into leaf protein is 5 : 1 for the sucrose experiments and 6 : 1 for the acetate experiments after 12 hours. The extent to which the experiments with N^{15} and C^{14} are comparable is, of course, uncertain. Under the conditions of the experiment the data demonstrate, however, that incorporation of amino acids proceeds at a considerably greater rate into protein of excised roots than into protein of excised leaves. In so far as incorporation may be said to be synthesis, it seems possible that the decrease in protein content of detached leaves is due in part, at least, to a lower rate of protein synthesis.

The author wishes to acknowledge the advice and assistance of Professor James Bonner and Dr. G. C. Webster during the course of this work.

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BRIEF PAPERS

THE FORMATION OF INDOLEACETYLASPARTIC ACID IN PEA SEEDLINGS[1,2]

W. A. ANDREAE AND NORMAN E. GOOD

SCIENCE SERVICE LABORATORY, UNIVERSITY SUB POST OFFICE, LONDON, ONTARIO

Although the morphological effects of the application of indoleacetic acid (IAA) to plants are well defined, very little is known of the fate of IAA after it has entered the plant tissues. An enzyme system which brings about the oxidation of IAA to unknown products has been described (4). Recently Siegel and Galston (5) have reported that IAA is incorporated into a protein complex by pea root tips. Andreae (1) working with pea stems and roots observed that exogenous IAA is largely converted in the tissue into an ether-insoluble substance which gives a stable mauve color with the Salkowski reagent ($FeCl_3$-H_2SO_4) and is not affected by the IAA oxidase of peas. This substance has now been identified as indoleacetylaspartic acid.

The ether-insoluble compound was extracted from Alaska peas which were grown in darkness for six days, then exposed to red light for two hours and harvested the following day. The stems were sectioned in to 2-inch pieces and floated with continuous gentle shaking in a solution containing 30 ppm IAA in M/60 NaH_2PO_4. After 24 hours the stem sections were removed from the solution and thoroughly washed. Eight hundred grams of the treated stems were then macerated with 200 ml of 0.1 *N* $NaHCO_3$ in a Waring Blendor. The resulting brei was filtered through cheese cloth, centrifuged and extracted with 100 ml of peroxide-free ether. The pH was adjusted to 4.0 with phosphoric acid and the ether extraction repeated three times. The clear aqueous phase was further acidified with H_3PO_4 to pH 2.6 and then extracted three times with n-butanol. The n-butanol was washed once with a small volume of water and then extracted twice with small amounts of 0.1 *N* $NaHCO_3$ solution. The bicarbonate solution was acidified to pH 2.6 with H_3PO_4 and extracted with a minimum volume of n-butanol (about 5.0 ml). This butanol extract was transferred to a column of powdered Whatman No. 1 filter paper which had been washed with a mixture of isopropanol (80 parts), concentrated NH_4OH (10 parts) and water (20 parts). The column was then eluted with the same isopropanol-ammonia mixture. The Salkowski-positive material came off in a single band. The solvent was removed by distillation at reduced pressure and the residue was taken up in a small volume of water.

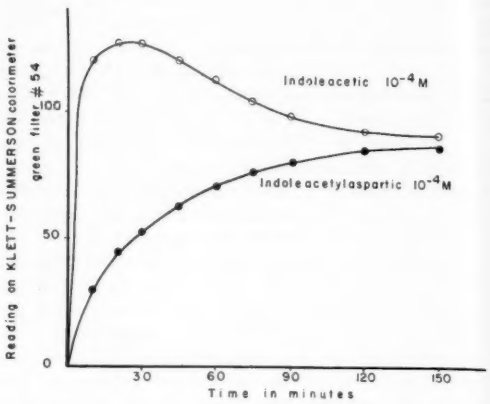

FIG. 1. One ml of 10^{-4} M indoleacetic acid (IAA) or indoleacetylaspartic acid mixed with 4 ml of Salkowski reagent (15 ml 0.5 M $FeCl_3$, 300 ml conc H_2SO_4, 500 ml H_2O). Color measured with Klett-Summerson colorimeter using green filter No. 54.

The resulting solution produced a mauve color with the Salkowski reagent which attained half of its full intensity in 20 minutes and full intensity after 2 hours. Under the same conditions IAA produced a pink color which reached maximum intensity within 20 minutes (fig 1). The U.V. absorption of IAA and the ether-insoluble material corresponded closely, each having the maxima at 280 and 288 mμ characteristic of the indole nucleus. An aqueous solution of the ether-insoluble material withstood boiling for several hours and was stable for many days in the refrigera-

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[2] Contribution No. 49, Science Service Laboratory, Canada Department of Agriculture, University Sub Post Office, London, Ontario, Canada.

TABLE I

COMPARISON OF PURIFIED PEA EXTRACT AND SYNTHESIZED INDOLEACETYLASPARTIC ACID

MATERIAL	R_f IN ISOPROPANOL-AMMONIA	CONC BASED ON ABSORPTION AT 280 mμ	IAA CONC IN HYDROLYZATE (SALKOWSKI COLOR)	ASPARTIC ACID CONC IN HYDROLYZATE (NINHYDRIN COLOR)	MOLAR RATIO IAA/ASPARTIC ACID IN HYDROLYZATE
Purified pea extract	0.07	1.9×10^{-2} M	1.95×10^{-2} M	1.8×10^{-2} M	1.08
Synthesized indoleacetylaspartic acid	0.07	5.75×10^{-2} M	5.4×10^{-2} M	5.9×10^{-2} M	0.92

tor. It resisted oxidation and hydrolysis by pea enzymes but was completely hydrolyzed in 1 *N* HCl or 7 *N* NaOH in less than 3 hours at 100° C. The hydrolysis products were IAA and an acidic amino acid chromatographically identical with aspartic acid (figs 2 and 3). After alkaline hydrolysis IAA and the amino acid were present in equimolar amounts (table I). Indoleacetylaspartic acid prepared by a modification of the method of peptide synthesis described by Boissonnas (2) was identical with the substance isolated from pea stems in all aspects studied; both gave similar mauve colors with the Salkowski reagent, had the same U.V. absorption maxima at 280 and 288 mμ, yielded the same products on hydrolysis (IAA and aspartic acid), had the same R_f when chromatographed on paper with isopropanol-ammonia (0.07) and showed the same activity in various biological tests. Indoleacetylasparagine, which would also yield equimolar amounts of IAA and aspartic acid on hydrolysis was likewise synthesized by the Boissonnas method. However, the asparagine derivative having only one free carboxyl moved much more rapidly on paper chromatograms developed with basic solvents (R_f 0.20 in isopropanol-ammonia).

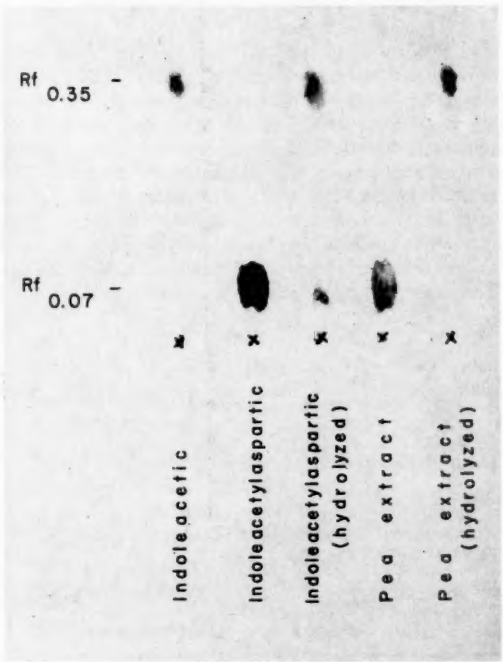

FIG. 2. Paper chromatogram on Whatman No. 1 filter paper developed with isopropanol-ammonium hydroxide-water (80 : 10 : 10) and sprayed with a mixture of 1 part 0.05 M ferric chloride and 50 parts of 5% perchloric acid to reveal Salkowski-positive areas. Hydrolyzates of indoleacetylaspartic acid and pea extract were prepared by heating to 100° C for 30 min in 6 *N* $Ba(OH)_2$, the $Ba(OH)_2$ being removed by treatment with solid carbon dioxide.

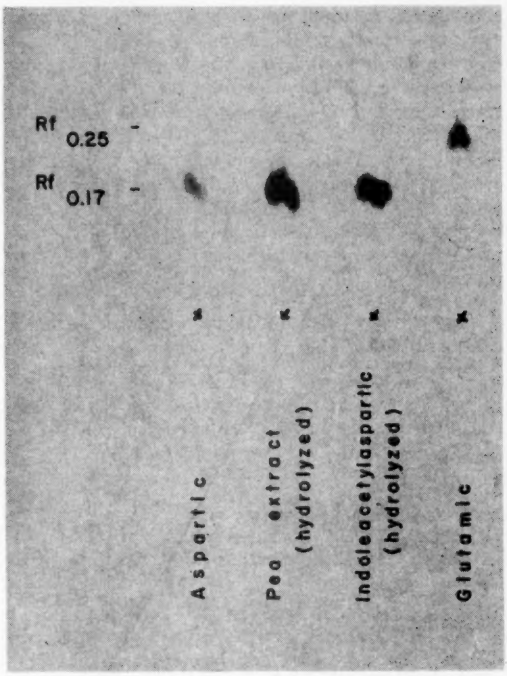

FIG. 3. Paper chromatograms on Whatman No. 1 filter paper developed with water saturated phenol plus 3% conc NH_4OH and sprayed with 0.1% ninhydrin in n-butanol. Hydrolyzates were prepared as under fig 2.

After incubation for 24 hours in a solution containing 30 ppm IAA, pea stems contained on a fresh weight basis about 15 ppm of IAA and 75 ppm of indoleacetylaspartic acid (45 ppm IAA equivalents). IAA treated excised pea roots contained similar proportions of free and conjugated IAA. Tissues which had not been incubated with IAA did not contain detectable amounts of either indoleacetic or indoleacetylaspartic acids. Barley coleoptiles incubated with IAA contained large amounts of IAA but no indoleacetylaspartic acid or other ether-insoluble, Salkowski-positive substances. Investigations with other plant species are in progress.

While there has been no previous report of the natural occurrence of amino acid derivatives of IAA, Jerchel and Staab-Müller (3) have prepared synthetically a number of indoleacetylated amino acids and have tested them for growth activity on slit pea stems. They found the glycine and cysteine derivatives to be practically inactive while the alanine derivative was as active as IAA. We have found that indoleacetylaspartic acid has less than 1/1000 the activity of IAA on slit pea curvature or pea stem elongation. Oat coleoptiles, however, respond to the complex as readily as to IAA in curvature and elongation tests.

The conjugation of various aromatic acids with

amino acids is a well known phenomenon in animals and has been interpreted as the detoxication of otherwise poisonous substances. The formation of indoleacetylaspartic acid is the first instance of a similar reaction in plants which has come to the attention of the authors. The significance of the reaction in the physiology of the pea is not yet understood. However, the marked difference between plant species with respect to the biosynthesis and utilization of indoleacetylaspartic acid suggests an approach to the problem of selective herbicidal action which may prove useful.

The authors wish to acknowledge the technical assistance of M. W. H. Van Ysselstein.

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INHIBITION OF PLANT GROWTH BY 2-MERCAPTOBENZIMIDAZOLE ANALOGS[1,2,3]

THEODORE L. REBSTOCK, CHARLES D. BALL, CHARLES L. HAMNER AND HAROLD M. SELL

Departments of Agricultural Chemistry, Chemistry and Horticulture, Michigan State College, East Lansing, Michigan

Galston et al (2) have reported that benzimidazole, a synthetic analog and metabolic antagonist of adenine, markedly inhibits cell elongation. These workers state that "the inhibition of cell elongation caused by benzimidazole is partially reversed by adenine, guanine, adenosine, guanosine, yeast adenylic acid and guanylic acid. This fact may implicate purine-containing compounds such as nucleic acids in the auxin-controlled growth reactions of plant cells." Recently Gillespie et al (3) have shown that 4-methoxy-6-nitrobenzimidazole was an effective growth inhibitor of *Tetrahymena gelii*, a guanine-requiring protozoan, and of developing embryos of *Rana pipiens*.

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The results of previous investigators (6) have shown that compounds physiologically active in cell elongation usually have a carboxyl group. By reacting 2-mercaptobenzimidazoles with an appropriately substituted acetic acid derivative, a carboxyl group can be attached to the benzimidazole nucleus. The resulting compound bears a structural relationship to 2,4-dichlorophenoxyacetic acid. Both of these substances possess an acetic acid residue attached to an aromatic nucleus through an ether linkage. The benzimidazole derivative contains a thiol ether bond ($-S-CH_2-\overset{\overset{O}{\|}}{C}-OH$) while the 2,4-dichlorophenoxyacetic acid has an oxygen ether bond ($-O-CH_2-\overset{\overset{O}{\|}}{C}-OH$).

Table I

Properties and Inhibition of New Leaf Growth of Cranberry Bean Plants by Several Derivatives of 2-Mercaptobenzimidazole

Compound	Melting point °C	Formula	Nitrogen % Calc'd	Nitrogen % Found	Inhibition **
2-Carboxymethylmercaptobenzimidazole	214–215 *	$C_9H_8O_2N_2S$	13.4	13.6	+
2-Carboxyethylmercaptobenzimidazole	178–179	$C_{10}H_{10}O_2N_2S$	12.6	12.6	0
2-Carboxy-(α-methyl)-methylmercaptobenzimidazole	181–182	$C_{10}H_{10}O_2N_2S$	12.6	12.6	0
5-Chloro-2-carboxymethylmercaptobenzimidazole	193–194	$C_9H_7O_2N_2SCl$	11.5	11.1	+++
5-Chloro-2-carboxyethylmercaptobenzimidazole	103–105	$C_{10}H_9O_2N_2SCl$	10.9	11.0	++
5-Chloro-2-carboxy-(α-methyl)-methylmercaptobenzimidazole	166–167	$C_{10}H_9O_2N_2SCl$	10.9	11.2	++

* Everett (1) reports the melting point as 190° C. Stephan and Wilson (4) give 215° C for the melting point of this compound.

** The number of plus signs indicate the relative magnitude of inhibition of new leaf growth as compared to non-treated plants.

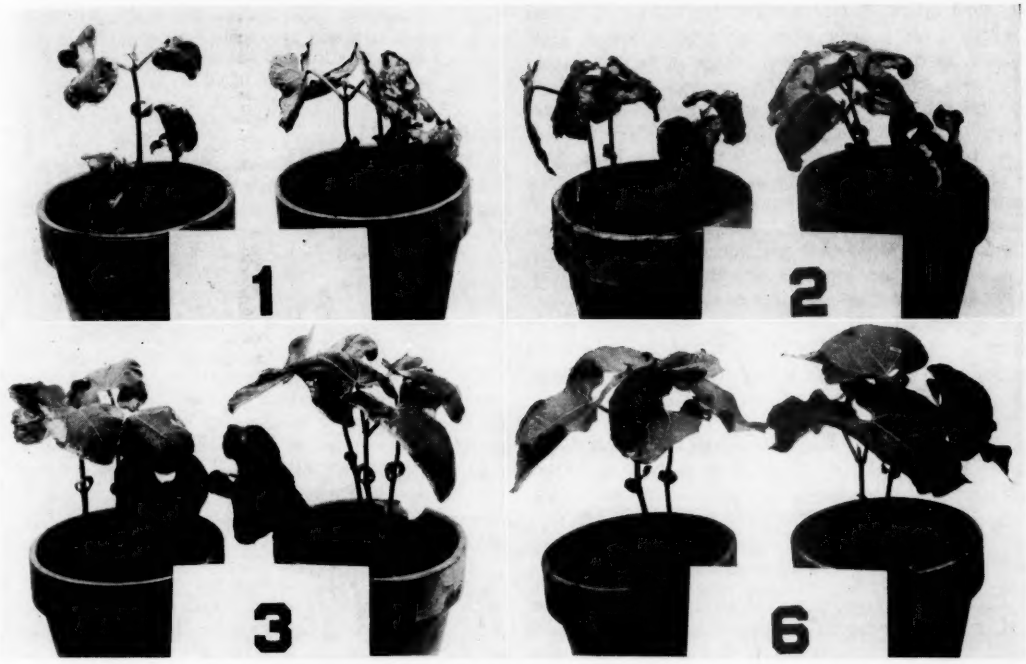
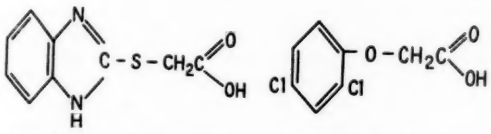


FIG. 1. Appearance of red kidney bean plants 6 days after treatment.
1. 5-chloro-2-carboxymethylmercaptobenzimidazole
2. 5-chloro-2-carboxyethylmercaptobenzimidazole
3. 2-carboxymethylmercaptobenzimidazole
6. Not treated.



2-Carboxymethylmercapto-benzimidazole

2,4-Dichlorophenoxy-acetic acid

In the present work several compounds related to benzimidazole were synthesized and tested as possible plant growth inhibitors. The 2-mercaptobenzimidazole derivatives were prepared by the procedure of Everett (1) in which the appropriate 2-mercapto-benzimidazole was reacted with monochloroacetic, α-bromopropionic or β-bromopropionic acids. The 2-mercaptobenzimidazole was supplied through the courtesy of the Monsanto Chemical Company, St. Louis, Missouri. The 5-chloro-2-mercaptobenzimidazole was prepared from 4-chloro-*o*-phenylenediamine utilizing the method described by Van Allen and Deacon (5). The melting points and nitrogen analyses for these compounds are listed in table I.

Biological assay consisted of dipping the expanding primary leaves of cranberry bean seedlings grown in the greenhouse into a 0.005 M solution of the compound. The inhibition of new leaf growth was used as an index to evaluate the growth activity of each compound as compared to non-treated plants (table I). The benzimidazole derivatives producing the greatest inhibition of new leaf growth were those which had chlorine substituted in the 5 position. Following treatment of primary leaves of red kidney bean plants new growth became twisted and stunted and the leaves chlorotic (fig 1). At higher concentrations growth was completely inhibited followed by death of the plants.

TABLE II

ROOT LENGTH OF CUCUMBER SEEDLINGS 4 DAYS AFTER TREATMENT OF THE SEEDS WITH 0.005 M SOLUTIONS OF SEVERAL ANALOGS OF 2-MERCAPTOBENZIMIDAZOLE

TREATMENT *	LENGTH MM
Control	50
2-Carboxymethylmercaptobenzimidazole	11
2-Carboxy-(α-methyl)-methylmercaptobenzimidazole	11
2-Carboxyethylmercaptobenzimidazole	11
5-Chloro-2-carboxymethylmercaptobenzimidazole	6
5-Chloro-2-carboxy-(α-methyl)-methylmercaptobenzimidazole	8
5-Chloro-2-carboxyethylmercaptobenzimidazole	5

* Under similar conditions the root length of cucumber seeds treated with 2.4-D was 2.5 mm.

Root inhibition was also employed as an additional biological assay using cucumber seeds (variety Marketer) as the test material. Seeds were germinated at room temperature (approximately 25° C) under laboratory conditions of alternating light and darkness on filter paper impregnated with a 0.005 M solution of the compounds in Petri dishes. Four days after treatment the length of the primary roots was measured (table II), and root length was used as an index of growth inhibition. The roots that appeared from treated seeds were without root hairs. As was the case in the previous experiment, the chlorine substituted benzimidazoles were the most active.

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A SURVEY OF THE NONVOLATILE ORGANIC ACIDS IN SEEDLINGS OF SOME GRASSES AND LEGUMES[1]

RAYMOND W. HOLTON and CLIFFORD R. NOLL, JR.
Department of Biological Chemistry, University of Michigan, Ann Arbor, Michigan

Isolated observations of organic acids in barley, oat, white lupine, and pea seeds and seedlings have been made over a number of years. Citric acid has been reported in etiolated barley and oat seedlings by Munch-Petersen (10). Täufel and Krusen (14) found citric acid in barley seeds and in green barley seedlings. In 1931 Nelson and Mottern (11) reported several organic acids in barley and oat, but they used mature green plants, not seedlings, for their investigations. While the research reported here was in progress, Elliott (6) demonstrated by means of paper chromatography the presence of citric, fumaric, glycolic, malic, malonic and succinic acids plus traces of cis-aconitic and α-ketoglutaric acids in etiolated barley seedlings. Using paper chromatography, Meiss (9) found only citric and malic acids plus an unidentified acid in etiolated white lupine seedlings. Only citric acid was shown to be in the ungerminated seed. Several organic acids have been identified in peas which have grown beyond the seedling stage (13, 17) and indoleacetic and pyruvic acids have been found in etiolated seedlings (1, 17). Citric acid has been reported in the ungerminated seed (10).

The present study is a survey of the normal distribution of non-volatile organic acids in seeds and etiolated and green seedlings of barley, oat, white lupine, and pea.

Extracts of the ungerminated seeds and of the aerial portions of five- to seven-day-old seedlings of the following species and varieties were chromatographed: *Hordeum vulgare*, var. Oderbrucker (barley), *Avena sativa*, var. Clinton (oat), *Lupinus albus* (white lupine), and *Pisum sativum*, var. Alaska (pea). In order that a quantitative comparison could be made between extracts, all chromatograms were made with a volume of the extract equivalent to 10 mg dry weight of tissue. The percent moisture was obtained by drying the tissues at 100° C for at least 48 hours.

Seeds were ground in a Wiley mill and then extracted with 90 % ethanol. Aerial portions of seedlings were first frozen in a dry ice–acetone mixture and then extracted with 90 % ethanol in a Waring blendor. After removal of the ethanol-insoluble material by filtration, the extract was diluted with two volumes of water. The solution was passed through an IRA–400 resin column to adsorb organic acids and anions (3). The column was washed with dilute ethanol and the adsorbed materials were eluted with 1 N $(NH_4)_2CO_3$. Elution was considered complete when the pH of the eluate increments remained the same as that of 1 N $(NH_4)_2CO_3$. Columns were regenerated with Na_2CO_3 rather than NaOH since IRA–400 columns in the OH^- form have been shown to convert sugars to lactic, glycolic, and other unidentified acids (12). The eluate was concentrated almost to dryness under reduced pressure at temperatures not exceeding 25° C and then dissolved in 50 % ethanol.

The 50 % ethanol solutions were analyzed by two-dimensional paper chromatography on Whatman No. 1 filter paper using the ascending technique. Two sets of chromatograms were run with each extract. On one chromatogram the first solvent phase was absolute ethanol : concentrated ammonia : water, 80 : 5 : 15, (5) and the second was n-butanol : formic acid, 95 : 5, saturated with water (8). On the second chromatogram an n-propanol : ethanol : 1.6 N $(NH_4)_2CO_3$, 10 : 60 : 30, phase (16) was followed by n-pentanol : 5 M formic acid phase (4). All ratios given are volume : volume. Both acidic solvents were allowed to age at least three weeks before use so that equilibrium between the acid and the alcohol and their ester could be obtained. Acid spots were located

[1] Received April 2, 1955.

by spraying the papers first with 1 % aqueous glucose and then with $AgNO_3$–NH_4OH solution according to the procedure of Gore (7).

Identification of spots was made by comparing their R_f values with those obtained in the same solvent systems for the following acid anions: cis-aconitate, adipate, citrate, fumarate, gallate, glutarate, glycerate, glycolate, indoleacetate, isocitrate, α-ketoglutarate, lactate, malate, maleate, malonate, oxalate, pyruvate, succinate, tartrate, and tricarballylate. The size of the spot was used to estimate the amount of acid present by comparison with standards. Known samples with 5 μgm or more of acid gave detectable spots. R_f values were determined also for the following inorganic anions: chloride, nitrate, phosphate, and sulfate.

Table I summarizes the acid anions detected in the various plant extracts. Unlisted anions for which R_f values have been determined were not identified in the extracts. Amino acids were also present on the chromatograms. They were located by a ninhydrin spray technique but were not identified. Each seedling had from one to six additional unidentified spots which were probably organic acids.

These data confirm the presence in etiolated barley seedlings of all the organic acids which Elliott (6) reported except fumaric acid, which she found in low concentration, and cis-aconitic and α-ketoglutaric acids, which were present in trace amounts. In addition, however, these data show that oxalic acid is also present. Oxalic acid, which has been suggested as an end product of plant metabolism, is the only organic acid which was found in all seed and seedling extracts.

Every seedling contained one or more of the Krebs tricarboxylic acid cycle acids and malic acid was found in all seedling extracts. The function of malonic acid is unknown. Although it is a competitive inhibitor of succinic acid dehydrogenase, its locus in the cell may be such that it does not interfere with Krebs cycle oxidations. Bentley (2) has reported the presence of malonic acid in mature legumes.

Tolbert and Cohan (15) have reported that glycolic acid oxidase, which is normally found in green tissues, is absent or in low concentration in etiolated grasses. They found that in etiolated tissues glycolic acid oxidase formation is activated by its substrate, glycolic acid. However, these data show that glycolic acid is present in all etiolated seedlings, sometimes in higher concentrations than in the green seedlings.

The normal distribution of organic acids in seeds and etiolated and green seedlings of barley, oat, white lupine, and pea has been studied. Citric and/or isocitric, glycolic, malic, malonic, and oxalic acids were found in the extracts and thirteen others were either absent or at levels not detectable by paper chromatographic techniques. Oxalic acid was present in all

TABLE I

DISTRIBUTION OF ACIDS IN SEEDS AND SEEDLINGS

PLANT PART	CITRATE AND/OR ISOCITRATE	GLYCOLATE	MALATE	MALONATE	OXALATE	SUCCINATE	CHLORIDE	NITRATE	PHOSPHATE
				Barley					
Seed	....	....	....	....	(**)	....	***	....	(*)
Seedling,									
etiolated	(*)	**	****	*	***	*	****	....	***
green	....	(*)	****	(*)	***	*	****	....	**
				Oat					
Seed	....	....	....	....	*	....	***	....	(*)
Seedling,									
etiolated	....	**	***	(*)	***	*	****	....	***
green	....	(*)	***	....	***	*	****	....	**
				Lupine					
Seed	....	....	....	....	**	....	**	....	*
Hypocotyl,									
etiolated	*	*	****	***	***	(*)	****	....	**
green	**	*	****	***	***	*	***	....	***
Cotyledon,									
etiolated	(**)	*	***	***	**	*	***	....	*
green	**	*	*	***	***	*	*	....	**
				Pea					
Seed	....	....	(*)	....	*	(*)	***	....	(**)
Seedling,									
etiolated	....	*	****	....	****	(*)	****	**	***
green	....	**	**	....	***	(**)	****	****	**

The number of asterisks indicates the approximate amount of each acid present in 10 mg dry tissue as follows: *—5 to 30 μgm, **—30 to 60 μgm, ***—60 to 120 μgm, ****—120 to 200 μgm. Parentheses indicate that the acid anion was identified on only one of the two 2-dimensional chromatograms.

seed and seedling extracts and malic acid was identified in all seedling extracts. Inorganic acid anions were also identified.

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TRANSPIRATION AND THE ABSORPTION AND DISTRIBUTION OF RADIOACTIVE PHOSPHORUS IN PLANTS[1,2]

KENNETH E. WRIGHT and NANCY L. BARTON

Department of Botany, Smith College, Northampton, Massachusetts

After a century of investigation the relationship of transpiration to the absorption and distribution of mineral salts in plants is still a controversial question. Evidence has been presented which supports the idea that these processes are largely independent while other researches have indicated enhanced absorption with increased transpiration. This lack of agreement among earlier investigators is brought out in the literature reviews and the experimental work of Hasselbring (3), Mendiola (7), and Muenscher (8). More recently an exhaustive set of experiments and an extensive review of the literature has been published by Hylmo (5). His results showed that in most species of plants tested the roots were strongly dependent on water transport for their supply of ions, and in all species the ion uptake by the stem and leaves was directly proportional to the water transport. The research reported in this paper was performed to determine the effects of transpiration upon the absorption and translocation of radioactive phosphorus in the sunflower plant, using the autoradiogram technique.

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[2] New Series Publication No. 61.

Seeds of the Mammoth Russian variety of sunflower were planted in sand and at the age of two weeks the seedlings were transferred to a complete culture solution. Culture solutions were changed twice a week, and daily supplements of distilled water were added to keep the solution at the top level. At the age of one month the plants were transferred to 500-ml bottles. For four days previous to the experimentation with P^{32}, when the plants were two months old, a record of the daily rate of transpiration was kept, and plants having approximately equal rates of water loss were selected for use. A complete culture solution containing 275 μc P^{32}/500 ml was placed in each culture bottle, plants inserted, and allowed to remain in contact with the P^{32} for 90 minutes, two under ordinary greenhouse conditions with one in light and the other in darkness, and a third in a high humidity chamber in light. Another two plants under greenhouse conditions in light had sucrose added to the culture solution such that the osmotic pressures of the culture solutions were 2.24 atms and 3.83 atms as determined by the cryoscopic method. The osmotic pressure of all of the culture solutions without added sucrose was 0.47 atms. During the 90-minute period all of the plants were in air kept in constant

motion by an electric fan. Relative humidity was computed from the readings on a wet and dry bulb thermometer.

At the end of the 90-minute run water loss was measured and then one leaf from each pair of leaves was cut from a plant, the leaves from each plant placed between blotters and dried under pressure for two hours. The leaves were then placed on a piece of herbarium paper, covered with a 20×25 cm sheet of Kodak No-Screen x-ray film and another sheet of herbarium paper, pressed under a weighted board, and exposed to the film for 14 hours. Films were developed with Kodak x-ray developer and fixer, and exposed to the photographic paper for 0.5 second. The distribution of radioactive phosphorus in the leaves is recorded in figure 1.

These autoradiograms indicate that there is a positive relationship between the rate of transpiration and the absorption of P^{32} and its translocation to the leaves of the plants, higher rates of transpiration being associated with greater amounts of P^{32} in the leaves. During the course of the experiment a Geiger counter was used to detect the presence of P^{32} in plants A and B, leaves at the top of plant A and at the bottom of plant B being the first to register on the counter. The autoradiograms of these plants show higher concentrations of P^{32} from top to bottom of plant A and from bottom to top of plant B. It looks as if a rapidly moving transpiration stream carried the phosphorus past the leaf traces of the lower leaves of the high transpiring plant, and a slowly moving transpiration stream allowed phosphorus to enter the lower leaf traces of the low transpiring plant. Hanson and Biddulph (2) set out to determine if there actually is a diurnal fluctuation in the amount of ions absorbed by the root or translocated to the xylem, and to determine if possible the cause of any noted variation. They found that plants in the daytime translocated four times as much radioactive rubidium to the shoots as plants in the nighttime. This increased daytime absorption and translocation of ions was attributed to some inherent capacity of the root symplast which is dependent upon an adequate supply of metabolites, a low salt–high sugar status, or possibly some unknown factor associated with illumination. They did not mention the possible effect of increased transpiration rate during the day. The difference in the amount and distribution of P^{32} in plants A and B could be affected by any of the factors postulated by Hanson and Biddulph since daylight and night were variables. However, it would seem foolhardy to rule out the influence of the differing rates of transpiration.

Plant C with a transpiration rate reduced by higher humidity shows a relatively low concentration of P^{32} when compared with plant A. In this case both plants were in light and humidity was the variable, thus leading to the conclusion that differences in P^{32} content could be attributed to difference in transpiration rates. That light might, under certain conditions, have some influence on translocation may be inferred from a comparison of plants B and C where the experimental variables were light and relative humidity. Although the rates of transpiration in these plants were about the same, the pattern of distribution of P^{32} was quite different.

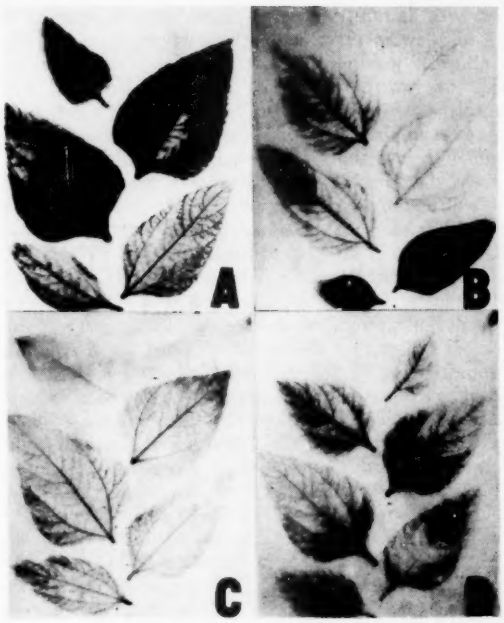

FIG. 1. Autoradiograms demonstrating the distribution of P^{32} in sunflower leaves arranged as they occurred on the plant under conditions of:

A. Water loss 1.5 gm/dm^2 × hr, 0.47 atm O.P., 67° F, 59% R. H., Light.
B. Water loss 0.6 gm/dm^2 × hr, 0.47 atm O.P., 69° F, 60 % R. H., Darkness.
C. Water loss 0.4 gm/dm^2 × hr, 0.47 atm O.P., 67° F, 95 % R. H., Light.
D. Water loss 1.3 gm/dm^2 × hr, 3.83 atm O.P., 67° F, 59% R. H., Light.

Plant D with a transpiration rate slightly reduced by a culture solution of 3.83 atms osmotic pressure (O.P.) showed a decrease in P^{32} content, a decrease greater than might have been anticipated with the degree of water loss reduction involved. The plant wilted considerably before the termination of the run, indicating that a portion of the water loss came from the plant tissues and not from the culture solution, thus largely cancelling the effects of a transpiration stream which could carry P^{32} from the roots to the leaves. The plant placed in a culture solution of 2.24 atms osmotic pressure is not recorded in figure 1. In this case the water loss was the same as from plant A in a culture solution of 0.47 atms osmotic pressure, and it was difficult to distinguish any differences in the autoradiograms.

The use of sucrose in the culture solution may be open to some criticism. That higher osmotic pres-

sures of culture solutions obtained by the addition of sucrose bring about a marked reduction in the entrance of water into a plant has been demonstrated by Tagawa (11), Rosene (9), and Hayward and Spurr (4). According to Long (6) the addition of sucrose to the culture solution greatly reduced water entrance into the roots of tomato plants but did not greatly disturb mineral entrance. Spoehr (10) and Went and Carter (12) found that sucrose was not absorbed through roots and its effect on growth was negligible. In direct contrast Dormer and Street (1) studying carbohydrate nutrition of tomato roots postulated a phosphorylation of sucrose with consequent entrance into plant cells. Hanson and Biddulph (2) studied the effect of added sucrose on the translocation of absorbed ions. They found that in darkness the addition of sugar to the culture solution had no significant effect on the translocation of phosphate to the shoots, but that in the daytime the translocation of phosphate did increase. This latter phenomenon was attributed to the increase in photosynthetic sugar and not to the sugar in the culture solution. The autoradiograms obtained from the plants whose roots were in contact with sucrose in the experiment here recorded show no enhanced absorption of phosphorus. Quite to the contrary, there is a reduced amount of P^{32} in the leaves of these plants, a condition attributed to a lack of transport of P^{32} to the tops of the plants because of a reduction in water flow through the xylem.

After two months of growth in a complete culture solution sunflower plants were transferred to culture solutions containing 275 μc P^{32}/500 ml and allowed to remain in contact with the P^{32} for 90 minutes. Transpiration was varied by light vs darkness, different relative humidities, and different osmotic pressures of the culture solution obtained by the addition of sucrose. The distribution of P^{32} in the leaves was recorded by means of autoradiograms. It was noted that whatever means was used to vary the amount of transpiration, and, therefore, the amount of water absorbed and transported upward through the plants, there was a positive relationship between water transport and the amount of P^{32} accumulation in the leaves, higher rates of water loss being associated with greater amounts of P^{32} in the leaves.

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STUDIES ON NITRITE METABOLISM IN HIGHER PLANTS[1]

STEVE VANECKO[2] AND J. E. VARNER

Agricultural Biochemistry Department, The Ohio State University, Columbus 10, Ohio

The theory that nitrate reduction proceeds in a stepwise manner as follows

$$NO_3^- \rightarrow NO_2^- \rightarrow HNO \rightarrow NH_2OH \rightarrow NH_3$$

has received much support recently with the isolation of nitrite reductase by Evans and Nason (2), the isolation of hydroxylamine reductase by Zucker and Nason (7) and the demonstration of a nitrite reductase system which reduces nitrite to ammonia (4). In this report manometric experiments with nitrite and experiments with $N^{15}O_2^-$ are presented to show further evidence that nitrite is an intermediate in nitrate reduction.

During manometric studies to determine the effect of nitrite on the processes of respiration and photosynthesis it was observed that leaves infiltrated with nitrite and exposed to light rapidly evolved a gas and caused a simultaneous disappearance of nitrite. Investigation showed that the gas was almost entirely oxygen and that the amount of oxygen evolved was

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[2] Charles F. Kettering Foundation Predoctoral Fellow.

TABLE I

FATE OF NITRITE SUPPLIED TO WHEAT LEAVES

	MICROMOLES	%
$N^{15}O_2^-$ taken up	37.0	100.0
$N^{15}O_2^-$ unchanged	1.0	2.7
$N^{15}O_2^-$ reduced to amino level	30.5	82.5

directly proportional to the amount of nitrite disappearing.

To determine the fate of the nitrite supplied to wheat leaves the following experiments were performed. One-gram samples of wheat leaves from 10- to 12-day-old wheat seedlings were placed with the cut ends in 10 ml of 0.01 M $KN^{15}O_2$. The $KN^{15}O_2$ was synthesized from $KN^{15}O_3$ according to a standard procedure for reducing nitrate to nitrite (3). The wheat leaves were exposed to light (tungsten filament) of 5000 fc intensity for four hours at 30° C, with air flowing rapidly over them. The leaves were extracted with hot 80 % ethanol, and nitrite and nitrate determined together by the method of Varner et al (6). Nitrite was determined in the original solution to establish the amount of $N^{15}O_2^-$ entering the leaves. Reduced nitrogen fractions (ammonia, amide, amino acid and proteins) were recovered in one sample by the Kjeldahl procedure. The ammonium sulfate from each of these three samples was converted to gaseous nitrogen by the method of Rittenberg (5) for determination of N^{15} by the mass spectrometer. Table I shows the results from these experiments.

These results clearly show that wheat leaves can reduce nitrite to the amino level of reduction. Since we have never observed a significant increase in the levels of ammonia and amides in the wheat leaves during the reduction of nitrite, the major part of the reduced nitrogen is probably being used in protein synthesis. It is of course understood that although a plant may be able to reduce nitrite, the normal reduction of nitrate by that plant does not necessarily proceed through the nitrite stage. However, the isolation of an enzyme capable of reducing nitrate to nitrite (2) lends support to this hypothesis.

It is interesting to note in table I that very little nitrite accumulates in the wheat leaves, that is, it is reduced as rapidly as it enters the leaf. This is in contrast to the behavior of nitrate which will enter excised wheat leaves much more rapidly than it is reduced (1). It is difficult to say whether this difference in rates of reduction is due to a difference in rates at which the two ions can arrive at the reduction site or is due to a difference in capacities of the enzymatic systems involved.

TABLE II

CORRELATION BETWEEN NITRITE DISAPPEARANCE AND GAS EVOLUTION

NITRITE DISAPPEARED	GAS EVOLVED	MOLE RATIO
micromoles	*micromoles*	
2.3	3.1	1.34
2.4	3.5	1.46

TABLE III

EFFECT OF ETIOLATION ON LIGHT * REDUCTION OF NITRITE

LEAVES	CHLOROPHYLL CONTENT	NITRITE REDUCED
	mg/gm	*micromoles/gm*
Green	2.47	0.72 **
Etiolated	0.0	0.04 **
Etiolated + 2 hrs light	0.18	0.70 **

* Intensity, 500 fc.

** Each value represents the average of 2 or 3 determinations.

The quantitative relationship between the nitrite disappearing and the gas evolved during the illumination of wheat leaves was established in the following manner. A one-gram sample of wheat leaf sections (1 cm long) was vacuum infiltrated with 0.05 M KNO_2 and divided into two equal parts. One part was placed in a Warburg flask and illuminated for 20 minutes at 30° C, at 2000 fc. The other part of the sample was used as a dark control. The rate of reduction in the dark was usually less than 5 % of the rate in the light. The difference between the quantities of nitrite reduced in the light and the dark was used in calculating the ratio of nitrite disappearing as gas evolved. Typical data are shown in table II. Standard gas analysis tests showed that the gas evolved was oxygen with a little nitrogen in it.

If nitrite were reduced by the overall reaction

$$2HNO_2 + 2H_2O \rightarrow 2NH_3 + 3O_2$$

the ratio of oxygen produced to nitrite reduced would be 1.5. From the data in table II it can be concluded that the photolysis of water is the primary and immediate source of reducing substance used in the light reduction of nitrite by wheat leaves.

In experiments similar to these, wheat leaves were infiltrated with $KN^{15}O_2$ and the gas evolved analyzed for N_2^{15}. This gas always contained some N_2^{15} and in one experiment contained as much as 18 % N_2^{15}. Presumably this results from the reaction of nitrite with the amino nitrogen of the leaves.

The dependence of the light reduction of nitrite (measured by nitrite disappearance) on chlorophyll is shown in table III.

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FLOWER-PROMOTING ACTIVITY OF PEA SEED DIFFUSATES[1,2]

HARRY R. HIGHKIN

EARHART PLANT RESEARCH LABORATORY, DIVISION OF BIOLOGY,
CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

It has been established that certain varieties of peas can be vernalized (3). Cold treatment of the germinating seeds of such pea varieties induces a number of changes in the subsequent growth of the seedlings at normal temperature. Of these changes one of the most interesting is an acceleration of flowering. The physiological mechanisms by which such cold treatment affects the seed have, however, remained obscure. In this paper it will be shown that an extract may be prepared from pea seeds which may be used to replace low temperatures in the treatment of other seeds. This extract, like low temperatures, is effective in reducing the number of nodes to the first flower in non-cold treated but vernalizable pea varieties.

Pea diffusates of seeds were prepared by a modification of the method of Bonner, Haagen-Smit and Went (1): one end of a glass cylinder 5 cm long and 7.5 cm in diameter was wrapped in gauze and put into a 600-ml beaker. The beaker and the cylinder, immersed in glass distilled water, were covered with paper toweling and sterilized. Dry seeds were sterilized by immersion for 10 min in a 0.05 % solution of sodium hypochlorite.

They were then washed four to six times with sterile distilled water. About 80 sterile seeds were next put on the gauze and sufficient water put in the beaker so that when the seeds had become completely turgid they were partially immersed in the remaining liquid. The beakers were covered with paper toweling. The beakers containing the seeds were then subjected to the following conditions: 1) 4° C in dark for 25 days; and 2) 23° C in dark for 5 days. At the end of these treatment periods, the germinating seeds were removed and planted. Most of these plants did not survive; those that did, were damaged and could not be examined for flowering. The liquid (diffusate) which remained in the beakers was used for soaking fresh dry seed. The period of soaking in the diffusate was approximately 10 hours at 20 to 23° C. The plants after soaking were grown under conditions of a 24-hour cycle made up of eight hours natural light at 20° C, eight of artificial light at 17° C, and eight hours dark at 17° C.

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TABLE I

EFFECT OF PEA DIFFUSATES ON THE SUBSEQUENT FLOWERING OF PEAS

TREATMENT	MEAN NODE TO FIRST FLOWER
Control (H_2O)	20.2 ± 0.54
4° C Diffusate	18.4 ± 0.828 *
23° C Diffusate	17.85 ± 1.34 *

Diffusates made at low (4° C) or high (23° C) temperatures. Seeds soaked for 10 hrs in these diffusates or in distilled H_2O as the control and then grown at high temperature (20° C day, 17° C night).
* Differences from control significant at the 1 % level.

Table I shows the data of one of a series of experiments in which dry seeds were soaked either in the diffusate or in water. It can be seen that both diffusates, i.e., from 4° C (vernalized) and 23° C (unvernalized) seed, are effective in reducing the number of nodes to the first flower.

The data show that the diffusate contains an active principle capable of replacing cold treatment for this variety of pea which is normally quantitatively vernalizable. Diffusates were found to be inactive in the Avena section growth test, and it is therefore unlikely that the active principle is an auxin. It may however be a flowering hormone or possibly a precursor of a flowering hormone.

Earlier work, summarized by Fries (2) has shown that pea diffusates contain a substance or substances which causes appreciable growth of excised leaves. Removal of the cotyledons from otherwise normal etiolated pea seedlings resulted in a marked decrease in the subsequent growth of the leaves.

The present studies indicate the presence of still another factor in pea diffusates, namely a factor active in the promotion of flowering.

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The Midwestern Section: The Midwestern Section of the American Society of Plant Physiologists was organized at an invitational meeting of regional plant physiologists held June 13 and 14, 1955 on the campus of the University of Illinois. There were 143 registrants for the meeting. By-laws for the organization and a motion to petition the American Society of Plant Physiologists for recognition as a regional section were approved by unanimous vote. Officers elected were: *Chairman,* R. W. Howell; *Vice-Chairman,* H. Beevers; *Secretary-Treasurer,* J. B. Hanson.

The organization is open to all interested persons in the midwestern regions of the United States and Canada. The first formal meeting of the section is scheduled for next summer at Iowa State College, Ames, Iowa.—Aubrey Naylor.